

Chemostat enrichment and isolation of *Hyphomicrobium* EG
A dimethyl-sulphide oxidizing methylotroph and reevaluation
of *Thiobacillus* MS1

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Abstract. A stable mixed bacterial culture was obtained by chemostat enrichment using dimethyl-sulphoxide as a carbon and energy source. This culture could not only rapidly oxidize dimethyl-sulphoxide but also dimethyl-sulphide. Enzyme determinations indicated that an important part of it consisted of methylotrophs, which assimilated carbon via the serine pathway. Indeed plate counts revealed the majority of the community to be a *Hyphomicrobium* species. This organism, designated *Hyphomicrobium* EG, is an obligate methylotroph which can only grow aerobically on several different C₁-compounds. Its performance on dimethyl-sulphoxide was compared with that of the community and of another recently isolated strain, *Hyphomicrobium* S. The mixed culture, *Hyphomicrobium* EG and *Hyphomicrobium* S had a μ_{\max} of 0.08, 0.08 and 0.014 h⁻¹ respectively. The K_S for dimethyl-sulphoxide was the same for all three cultures (3–6 μ M), whereas that for dimethyl-sulphide of *Hyphomicrobium* EG after growth on dimethyl-sulphoxide was 3-fold higher than that of the other two cultures (48 and 16 μ M respectively). After growth on dimethyl-sulphide it improved to 3 μ M. Dimethyl-sulphide respiration was maximal at a concentration of 100 μ M; higher concentrations were inhibitory. One of the accompanying organisms, a pink methylotroph, was able to derive energy from the oxidation of thiosulphate. Available cultures of *Thiobacillus* MS1 that were reported to be able to utilize dimethyl-sulphide could no longer metabolize this compound.

INTRODUCTION

Dimethyl-sulphide (CH₃SCH₃, DMS) is a toxic (Ljunggren & Norberg 1943; Mhatre et al. 1983), malodorous and volatile compound with an odour threshold concentration of 2.5 parts per billion (Leach & Chung 1982). In the global S-cycle DMS, arising through cleavage of dimethyl- β -propiothetin, one of the products of algal sulphur metabolism (Bremner & Steele 1978; Andreae 1980), is assumed to play an important role in transporting reduced sulphur compounds from aquatic to terrestrial environments (Lovelock et al. 1972; Nguyen et al. 1978; Andreae & Raemdonck 1983). It is also one of the major compounds implicated in the volatilization of sulphur from soil (Banwart & Bremner 1976). Furthermore, high quantities of volatile sulphides are produced by the paper industry, in oil refineries, manures and sewer systems. As this may cause pollu-

tion problems, methods for the removal of these malodorous compounds are being investigated. Since DMS appears to be rather recalcitrant in waste purification (Sivelä & Sundman 1975; Imai 1983), it was decided to focus on its utilization by microorganisms.

In our hands, enrichments on DMS have been unsuccessful because of its toxicity. Earlier a *Hyphomicrobium* species was isolated which metabolized dimethyl-sulphoxide ((CH₃)₂SO, DMSO) via DMS to carbon dioxide and sulphuric acid (de Bont et al. 1981). The DMS-utilizer *Hyphomicrobium* S, which was obtained by batch enrichment on DMSO, was extremely difficult to cultivate and maintain because of its low μ_{MAX} on DMSO (0.014 h⁻¹) which hardly allowed physiological studies. This paper shows that by chemostat enrichment under DMSO limitation toxicity problems of DMS can be circumvented and relatively fast growing organisms can be obtained.

In the course of this study we also checked the DMS utilizing potential of available cultures of *Thiobacillus* MS1, an obligately chemolithotrophic *Thiobacillus* species which had been reported to utilize DMS as an additional carbon and/or energy source (Sivelä 1980).

METHODS

Organisms. *Hyphomicrobium* S was obtained from the Delft culture collection, number LMD 80.90. *Thiobacillus* MS1 was provided by Dr S. Sivelä, Helsinki. Another culture supposedly containing the same organism had been kept in liquid nitrogen in our own laboratory since 1976, under number LMD 80.66. Scrapings of a papermill biofilter used to purify waste water which contained methylated sulphur compounds were used as inoculum for a batch enrichment culture on DMSO as a carbon and energy source. This culture was filtered through a membrane filter with a pore width of 1.2 μm to remove protozoans and the chemostat was inoculated with the filtrate.

Media and culture conditions. The medium for chemostat enrichment and continuous cultivation of *Hyphomicrobium* EG and S contained (g l⁻¹): K₂HPO₄, 0.8; KH₂PO₄, 0.3; MgSO₄·7H₂O, 0.4; NH₄Cl, 0.4; DMSO, 0.78; and 2.0 ml of a trace element solution as described by Vishniac & Santer (1957), except that it contained 2.2 g of ZnSO₄·7H₂O instead of the original 22 g. A Biolafitte glass fermentor with a working volume of 1.5 l was used for the continuous flow enrichment. Culture conditions were: pH 7.0, pO₂ 50% air saturation, temperature 30 °C and D(ilution rate) 0.035 h⁻¹. Batch culture medium for *Hyphomicrobium* EG and S contained (g l⁻¹): Na₂HPO₄·7H₂O, 7.9; KH₂PO₄, 1.5; MgSO₄·7H₂O, 0.5; (NH₄)₂SO₄, 0.4; DMSO, 0.78; and 2.0 ml of above trace element solution. The pH was 7.2. Solid media were prepared by addition of

2% (w/v) Difco Bacto agar. Plate counts were done on batch culture medium to which 0.05% (w/v) yeast extract was added. The constituents of the mixed culture were isolated either on batch culture medium with or without added yeast extract, or on a rich medium called TY, using standard techniques. The latter contained (g l^{-1}): Difco tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0; the pH was adjusted to 7.2. All chemicals used were of analytical grade. The mineral and TY media were sterilized by autoclaving for 20 min. at 110 and 120°C respectively.

The chemostat and batch culture medium and the culture conditions for the pink methylotroph were the same as those for the *Hyphomicrobium* species except that DMSO was replaced by 10 mM Na-acetate plus or minus 2 mM Na-thiosulphate and that the pO_2 was 85% air saturation.

Media and culture conditions for *Thiobacillus* MS1 were comparable to those described by Sivelä (1980). To prevent DMS-losses from the culturing system (by diffusion and evaporation) silicon tubing was replaced by butyl rubber tubing, the medium was fed under the liquid level of the culture and the condenser was cooled with ice-water. The purity of the *Thiobacillus* species was checked by streaking on TY medium on which no growth should occur.

Respiration measurements. Maximum respiration rates were measured polarographically with a Clark-type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co., Ohio, USA) at 30°C. The methyl mercaptan (CH_3SH , MM) dependent oxygen uptake rate was recorded by adding gaseous MM to a culture sample. For this purpose an anaerobic jar was twice evacuated and filled with nitrogen gas before being filled with MM, to reduce its auto-oxidation. In these experiments unwashed samples from fermentor cultures were used, except for *Thiobacillus* MS1 cultures grown on mixtures of thiosulphate and DMS, which were washed with batch medium without thiosulphate prior to use. Rates are expressed in $\text{nmol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$ and were corrected for endogenous respiration and auto-oxidation. All data given are the means of at least three independent measurements.

Analytical procedures. DMSO and DMS were determined using a Varian 3700 gas chromatograph fitted with a 0.46 m \times 3 mm i.d. teflon column packed with acetone-washed Porapak QS and equipped with a flame photometric detector with an optical filter which permitted light transmission at 394 nm. Injection, column and detector temperatures were respectively: 200, 100 and 230°C. The gas flow rates to the dual-flame detector were 30 ml min^{-1} N_2 carrier, 80 ml min^{-1} air, 170 ml min^{-1} air and 140 ml min^{-1} H_2 . DMSO was first chemically reduced to DMS using stannous chloride in concentrated hydrochloric acid (Anness 1981) and subsequently determined as such. Sulphate was determined according to Dodgson (1961), formaldehyde according to Houle et al. (1970), for-

mate according to Battat et al. (1974), thiosulphate and tetrathionate according to Sörbo (1957), sulphide and sulphite as described by Trüper & Schlegel (1964), acetate with the Boehringer test-combination. Protein contents of whole cells and cell-free extracts were determined by the microbiuret method (Goa 1953), except that the cells were hydrolysed during one hour at 60°C instead of at 37°C. In the case of cell-free extracts the hydrolysis step in this method was omitted. Dry weights were determined by membrane filtration using filters with a pore width of 0.2 µm.

Preparation of cell-free extracts. Cells were harvested and washed twice with 50 mM potassium phosphate buffer pH 7.0 by centrifugation for 20 min. at 22,500 xg at 4°C. Approximately 0.5 g wet weight of cells was suspended in 1.5 ml 50 mM potassium phosphate buffer pH 7.0 containing 5.0 mM MgCl₂, and 0.75 g ballotini beads (0.11 mm diameter) was added to this suspension. Cells were disrupted by sonification for 5 × 30 s with a MSE 100 watt ultrasonic disintegrator. Whole cells and debris were removed by centrifugation for 30 min. at 40,000 xg at 4°C and the supernatant was used for enzyme assays. If RubP-Case activity was to be determined both the washing and sonification buffer were changed for a Tris(hydroxymethyl)methylamine buffer as has been described by Lorimer et al. (1977).

Enzyme assays. RubPCase was determined by measuring ¹⁴CO₂ fixation as described by Beudeker et al. (1980). All other enzyme determinations were done spectrophotometrically using a Pye Unicam SP 1800 with a temperature cuvette housing at 30°C. Hexulosephosphate synthase was determined according to van Dijken et al. (1978); hydroxypyruvate reductase according to Large & Quayle (1963); NAD(P)⁺-dependent formate dehydrogenase according to Patel & Hoare (1971); NAD(P)⁺-dependent formaldehyde dehydrogenase according to Stirling & Dalton (1978); DCPIP-dependent formaldehyde dehydrogenase according to Johnson & Quayle (1964); NAD(P)H-dependent DMSO reductase according to de Bont et al. (1981), except that the DMSO concentration used in the reaction mixture was 0.6 mM instead of 10.0 mM. Enzyme activities are expressed in nmol pyridine nucleotide (or DCPIP) oxidized or reduced per minute per milligram protein. All data given were the average of at least three independent measurements, and linearly proportional to the protein concentration.

RESULTS

Characteristics of the mixed culture

By enrichment in the chemostat at D(ilution rate) = 0.035 h⁻¹ on medium con-

Table 1. Rates of substrate dependent oxygen uptake by the DMSO-grown mixed culture. The culture was grown on 10 mM DMSO at $D = 0.035 \text{ h}^{-1}$. The final concentration at which the various substrates were tested is given behind each individual substrate.

Substrate	Concentration (mM)	Rate of oxygen uptake (nmol $\text{O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$)
Dimethyl-sulphoxide	0.1	803
Dimethyl-sulphide	0.1	854
Methyl-mercaptan	0.14	301
Sodium sulphide	0.05	484
Formaldehyde	3	180
Formate	1	38
Thiosulphate	10	207
Dimethyl-disulphide	0.12	70
Ethyl-sulphide	1	75
Methylamine	0.1-15	n.d.
Dimethylamine	3	29
Methanol	0.1-10	n.d.

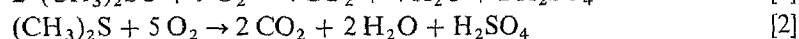
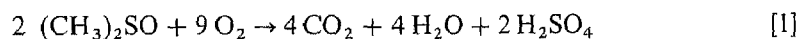
n.d. = not detectable

taining 10 mM DMSO as the only carbon and energy source a stable mixed culture was obtained, which could effectively metabolize DMSO. Neither DMSO, nor DMS, formaldehyde, formate and sulphide, the presumed intermediates of the metabolic pathways of DMSO (de Bont et al. 1981), were present at detectable levels in the culture liquid. From the alkali consumption of the culture it could be inferred that on utilization of 1.0 mmol DMSO 1.95 mmol acid was produced. The normal culture medium contained a relatively high sulphate concentration hindering the accurate measurement of sulphate produced by DMSO breakdown. Therefore sulphate determinations were done on the supernatant of the enrichment culture grown on a medium which contained DMSO also as the only sulphur source. These measurements indicated that more than 95% of the sulphur from DMSO had been converted to sulphate. These observations implied that most of the DMSO had been utilized. The dry weight and protein of the mixed chemostat culture on 10 mM DMSO at $D = 0.035 \text{ h}^{-1}$ were 176 and 72 mg l^{-1} respectively, and were doubled (365 and 151 mg l^{-1} respectively) on increasing the DMSO concentration in the medium feed to 20 mM. So DMSO was the growth limiting nutrient for this culture. Supply of additional DMS to the DMSO limited mixed culture gave an immediate increase in cell yield, suggesting that externally supplied DMS can effectually be metabolized by the mixed culture (data not shown).

To get an impression of the degradative potential of the mixed culture its

respiration rate on various substrates was determined (Table 1). The culture could respire DMS at a rate similar to that of DMSO. This indicated that also in this culture DMS might be an intermediate of DMSO metabolism. The maximal DMSO and DMS respiration rate was more than twice that necessary to grow on these compounds at $D = 0.035 \text{ h}^{-1}$. Indeed on gradually increasing the D to 0.08 h^{-1} washout occurred, whereas DMS became detectable when approaching this D , indicative of a build-up of intermediates of DMSO metabolism.

Complete oxidation of DMSO and DMS is according to:



Measurements carried out with an oxygen electrode to determine the quantity of oxygen required for the oxidation of limited amounts of DMSO indicated that the molar O_2/DMSO ratio was 2.6, whereas 4.5 mole oxygen would be required for the complete oxidation of 1 mole DMSO (Equation 1). This difference indicated that approximately 40% of the DMSO was assimilated by methylotrophs present in the culture (see also Table 2). This agreed with the yield of 0.23 gram dry weight/gram DMSO, which implied that about one third of the DMSO was assimilated, assuming that cells consist for 50% of carbon.

Constituents of the mixed culture

When initial attempts were made to isolate the different organisms from the mixed culture no growth was observed on DMSO containing agar plates. In order to get some clues on the physiological type(s) of organism present in the culture, the activity of key enzymes for methylotrophic and autotrophic growth was determined (Table 2). A high hydroxypyruvate reductase activity was obtained, indicative of the presence of methylotrophs utilizing the serine pathway for formaldehyde assimilation. Only a very low RubPCase activity was found (Table 2) which made it highly unlikely that an important part of the culture was growing autotrophically. On the basis of this information samples were restreaked on agar media containing a variety of C_1 -compounds (methanol, dimethylamine, methylamine, formate and DMSO with some yeast extract). Pinpoint colonies formed in about a weeks time on plates containing methylated amines or formate, whereas only after prolonged incubation (2–3 weeks) high viable counts could be obtained on DMSO containing agar plates to which 0.05% (w/v) yeast extract had been added. Plate counts indicated that 96% of the culture was made up of a *Hyphomicrobium* species, 2% was an orange colony forming organism, 1% a gram negative bacterium producing yellow colonies and the remaining 1% consisted of 3 different types of bacteria. The viability of the culture only amounted to 67%, due to (initial) difficulty of growing the

Table 2. Enzyme activities in cell-free extracts of the DMSO-grown mixed culture. The mixed culture was grown on 10 mM DMSO in a chemostat at $D = 0.035 \text{ h}^{-1}$.

Enzyme	(Coenzyme)	Activity ($\text{nmol min}^{-1} (\text{mg protein})^{-1}$)
Hexulosephosphate synthase		n.d.
Hydroxypyruvate reductase	(NADH)	2698
Hydroxypyruvate reductase	(NADPH)	17
Formaldehyde dehydrogenase	(NAD^+)	23
Formaldehyde dehydrogenase	(NADP^+)	n.d.
Formate dehydrogenase	(NAD^+)	278
Formate dehydrogenase	(NADP^+)	n.d.
Ribulose-bisphosphate carboxylase		3
Dimethyl-sulphoxide reductase	(NADH)	9
Dimethyl-sulphoxide reductase	(NADPH)	18

n.d. = not detectable

Hyphomicrobium species on plates. Of the purified bacteria only the *Hyphomicrobium* species, designated *Hyphomicrobium* EG, appeared to be able to grow on DMSO as the only carbon and energy source. Furthermore, the other isolates were unable to grow on intermediates of the metabolic pathway of DMSO. So *Hyphomicrobium* EG was solely responsible for the breakdown of DMSO by the mixed culture.

In a parallel enrichment culture on DMSO which had been inoculated with a sample from the same source but which did not have a pH control (its pH was daily adjusted which caused fluctuations from pH = 7.0–5.5 to occur) a pink facultative methylotroph made up 2% of the culture. It probably functioned in removing excretion products, possibly formaldehyde, of *Hyphomicrobium* EG at the lower pH. Interestingly, a pure culture of this organism was able to utilize thiosulphate mixotrophically. Dry weight, protein and total organic carbon of the culture grown on 10 mM acetate increased from 185; 51 and 99 mg l^{-1} respectively to 252, 68 and 110 mg l^{-1} respectively upon addition of 2 mM thio-sulphate. Furthermore, this methylotroph could oxidize sulphite at a rate of 80–110 $\text{nmol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$ regardless of how it had been grown. This might suggest a role in sulphite removal since pure *Hyphomicrobium* EG cultures were unable to oxidize this compound (unpubl. data).

Characteristics of Hyphomicrobium EG

Hyphomicrobium EG showed the typical morphology of the group of budding prosthecate bacteria. The size of the cells was $0.4\text{--}0.7 \times 1.2 \mu\text{m}$. *Hyphomicrobium* EG grew at pH-values ranging from 6.5 to 9.0 with an optimum at pH =

Table 3. Growth of *Hyphomicrobium* EG on several different compounds.

Dimethyl sulphoxide	+	Methylamine	+
Dimethyl sulphide	+	Dimethylamine	+
Dimethyl sulphone	-	Trimethylamine	±
Methanol	-	Trimethylamine-N-oxide	+
Formate	±	Ethanol	-
Thiosulphate	-	Acetate	-

+ = good growth; ± = weak growth; - = no growth

7.0–7.5. Its temperature optimum was 30 °C. It could use several C₁-compounds as carbon and energy source (Table 3) in contrast with *Hyphomicrobium* S which can only grown on DMSO and DMS (de Bont et al. 1981). Both *Hyphomicrobium* spp. were unable to grow anaerobically using nitrate, nitrite or fumarate as an electron acceptor. *Hyphomicrobium* EG could use methylated amines, nitrate and nitrite as nitrogen source and was unable to fix N₂.

Preliminary experiments indicated that an increase in yield of 5 mg protein/mmol thiosulphate was obtained when this organism was grown in batch culture on a mixture of thiosulphate and a C₁-compound as compared to the yield on the C₁-compound alone. This suggested that *Hyphomicrobium* EG is a chemolithoheterotroph (an organism which needs an organic compound as carbon and energy source but can derive extra energy from the oxidation of reduced inorganic substrates). Further details on the metabolism of this organism will be published elsewhere (Suylen et al., in prep.).

Comparison of *Hyphomicrobium* EG, S and the mixed culture

When *Hyphomicrobium* EG was grown at $D = 0.035 \text{ h}^{-1}$ on DMSO in a chemostat under the same conditions as the mixed culture, its yield and protein content were very similar to that of the enrichment culture. Its μ_{MAX} on DMSO was the same as that of the mixed culture and higher than that of *Hyphomicrobium* S (Table 4), which respired DMSO and DMS about three times slower

Table 4. K_S and μ_{max} of the mixed culture, *Hyphomicrobium* S and *Hyphomicrobium* EG.

The cultures were grown on 10 mM DMSO in a chemostat. For *Hyphomicrobium* S the dilution rate used was 0.014 h^{-1} , that for *Hyphomicrobium* EG and the mixed culture was 0.035 h^{-1} . K_S -values were determined from Lineweaver-Burk plots made with respiration rates measured with an oxygen electrode at various DMS(0) concentrations.

Culture	K_S (DMSO)	K_S (DMS)	μ_{max} (DMSO)
Mixed culture	4 μM	17 μM	0.08 h^{-1}
<i>Hyphomicrobium</i> S	5 μM	16 μM	0.014 h^{-1}
<i>Hyphomicrobium</i> EG	6 μM	48 μM	0.08 h^{-1}

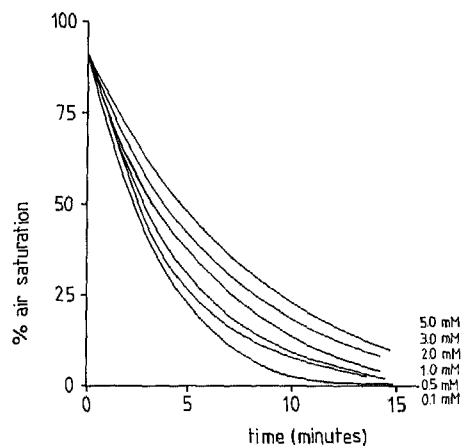


Fig. 1. Respiration of increasing concentrations of DMS by *Hyphomicrobium EG* as a function of time. The organism was grown in a DMSO-limited chemostat at $D = 0.035 \text{ h}^{-1}$.

than the mixed culture did. The substrate affinity constant (K_s) of the cultures for DMSO was comparable, whereas that of *Hyphomicrobium EG* for DMS was 3-fold higher (Table 4). Nevertheless *Hyphomicrobium EG* could easily be grown in a chemostat on 10 mM DMS as the only carbon and energy source at $D = 0.035 \text{ h}^{-1}$, after which a much lower K_s for DMS ($3 \mu\text{M}$) was found than after growth on DMSO, whereas the K_s for DMSO was comparable ($3 \mu\text{M}$).

On determination of the K_s -values of *Hyphomicrobium EG* for DMS the toxic effect of this compound became apparent. High concentrations of DMS gave immediate inhibition of respiration, whereas on standing the extent of inhibition increased even further (Fig. 1).

Revaluation of *Thiobacillus MS1*

The two available *Thiobacillus MS1* cultures appeared to be mixed cultures consisting of an obligately chemolithoautotrophic *Thiobacillus* sp., provisionally called *Thiobacillus O*, and several other (heterotrophic) organisms, unable to grow on thiosulphate, DMS or mixtures of these compounds. Like the described *Thiobacillus MS1* (Sivelä 1980), *Thiobacillus O* and the two mixed *Thiobacillus MS1* cultures could be grown in a chemostat under thiosulphate limitation (with or without vitamins), but no increase in yield was obtained upon addition of DMS, whereas DMS was invariably detectable at the air outlet of the fermentor. (Dry weights found on 30 mM thiosulphate with no or 1.5, 3.0 and 5.0 mM DMS added were 117, 114, 108 and 108 mg l^{-1} respectively).

After growth in the presence or absence of DMS the cultures were unable to oxidize DMS, and had a thiosulphate oxidizing capacity of 1100–1200 nmol

$O_2 \text{ min}^{-1} (\text{mg protein})^{-1}$, which is in the order normally found for obligately chemolithotrophic thiobacilli (Beudeker et al., 1982). DMS at 5.0 mM inhibited the thiosulphate respiration rate by only 10%, in contrast with an inhibition of DMS respiration found in *Hyphomicrobium* EG of about 40%. Cell-free extracts of the mixed (MS1) and pure *Thiobacillus* O cultures contained normal levels of RubPCase activity (184–248 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$; original data of Sivelä (1980) 8.6–15.0 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$), low hydroxypyruvate reductase activity (24–8 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$) as opposed to the activity given by Sivelä (1980) of 3.2–0 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ and no detectable hexulose phosphate synthase activity, indicating that these cultures were unable to assimilate carbon from DMS via the serine or the RuMP pathway. Isocitrate dehydrogenase activity found was 227–203 versus a published value (Sivelä 1980) of 17000–8500 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. Furthermore, in accordance with Sivelä's data, no formaldehyde- and formate dehydrogenase activities were found suggesting that these cultures could not dissimilate the carbon from DMS either.

DISCUSSION

By chemostat enrichment on DMSO a relatively fast growing *Hyphomicrobium* species was obtained. Its μ_{MAX} on DMSO was about five times higher than that of the previously described *Hyphomicrobium* S (de Bont et al. 1980), whereas the K_s for DMSO of both organisms after growth on DMSO was comparable (Table 4). *Hyphomicrobium* EG is an obligate, aerobic methylotroph with a much wider substrate range than *Hyphomicrobium* S, which only grows on DMSO or DMS. It is unable to grow on methanol or oxidize it after growth on DMSO. Therefore methanol is unlikely to be an intermediate in DMSO breakdown as it was found to be in monomethyl sulphate degradation (Ghisalba & Küenzi 1983) and the organism metabolized DMSO in the same way as *Hyphomicrobium* S did (de Bont et al. 1980). The DMSO reductase of *Hyphomicrobium* EG was mainly NADPH-dependent (unpubl. data), in contrast with that of *Hyphomicrobium* S (de Bont et al. 1980) and other bacterial strains (Bamforth 1980), but in accordance with the enzyme in yeasts (Anness et al. 1979; Bamforth 1980; Bamforth & Anness 1981). Chemostat cultures of *Hyphomicrobium* EG grown on DMS could still metabolize DMSO (witness the high affinity for this compound). This suggested that the enzymes for DMSO and DMS breakdown are regulated coordinately and/or synthesized constitutively.

The toxicity of DMS (Fig. 1) probably explains why enrichments by us and others (Y. A. Trotsenko, S. H. Zinder & T. D. Brock, personal communication) using this compound as the only substrate have been unsuccessful. These failures may partly be due to its (contrary to general belief, see for instance the Merck Index) rather high solubility in aqueous solutions amounting to about 300 mM

(Kushelev et al. 1976; Wood 1981; Przyjazny et al. 1983), which has led to the use of too high DMS concentrations. Thus chemostat enrichment using DMSO as the only carbon and energy source seems to offer a way to circumvent toxicity problems with DMS and makes the isolation of bacteria able to metabolize DMS easier.

Both the Y_{DMSO} of the mixed culture (17.6 g dry weight/mole) and the O_2/DMSO ratio found in experiments in which the respiration of limited amounts of DMSO was measured implied that approximately one third of the carbon from DMSO was assimilated. This was only natural if one realizes that the major part of the culture consisted of a (serine pathway utilizing) *Hyphomicrobium* species. Compared to the yield on methanol of other serine pathway utilizing methylotrophs (12.0–14.0 g dry weight/mole; Goldberg et al. 1976) the Y_{DMSO} was rather high, whereas these compounds should be energetically equivalent, given the metabolic pathway of DMSO (de Bont et al. 1980). Combined with the observation that the culture rapidly oxidized Na_2S this suggested that *Hyphomicrobium* EG derived energy from the oxidation of the sulphur moiety of DMSO, and hence would be a chemolithoheterotroph.

Although it seems obvious that the enrichment method used selects for an organism able to use both the sulphur moiety and the methyl groups of DMSO a community of organisms is also conceivable. For example the pink sulphite/thiosulphate oxidizing methylotroph in the chemostat culture grown without pH control could be growing on C_1 - or reduced-sulphur-compounds excreted by *Hyphomicrobium* EG under these stress conditions. Although a low RubP-Case activity was found in cell-free extracts of the mixed culture (Table 2) we were unable to isolate any *Thiobacillus*-like organism from it. Nevertheless one could imagine thiobacilli to be involved in DMSO breakdown as well. Whether one can reisolate a *Thiobacillus* MS1-like organism that can use the methyl groups of DMS as carbon but not as energy source (Sivelä 1980) remains to be seen. We have been unable to show DMS utilization by the available cultures of *Thiobacillus* MS1. Furthermore, a reevaluation of the original respiration- and enzyme data showed that incorrect units had been used. However, no systematic correction factor could be given (S.K. Sivelä personal communication). In view of our own results it seems therefore justified to conclude that the original (enzyme) data held insufficient proof for the functioning of the serine pathway of carbon assimilation in *Thiobacillus* MS1.

Finally, the existence of three typical C_1 -metabolizers namely two *Hyphomicrobium* species and a pink methylotroph which are unable to grow autotrophically but can derive energy from the oxidation of sulphide or thiosulphate, stresses the difficulty of drawing border lines between different physiological types of bacteria in the context of their taxonomic position. Bearing in mind that methylotrophs like *Paracoccus denitrificans* and *Thiobacillus versutus* (A_2) grow autotrophically on C_1 -compounds (Cox & Quayle 1975; Kelly & Wood

1982) and chemolithoautotrophically on thiosulphate (Friedrich & Mitrenga 1981; Gottschal & Kuenen 1980), one should expect that nature harbours a complete spectrum of bacteria with combinations of methylotrophic and chemolithotrophic capabilities.

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