Chemolithotrophic potential of a *Hyphomicrobium* species, capable of growth on methylated sulphur compounds

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Abstract. The yield of *Hyphomicrobium* EG on dimethyl sulphoxide, dimethyl sulphide and methylamine, considering the metabolic pathways of these compounds, suggested that the organism gained energy from the oxidation of the sulphur moiety of the former compounds. Indeed, a comparison of chemostat cultures of *Hyphomicrobium* EG grown on methylamine in the presence and absence of sulphide or thiosulphate proved this obligate methylotroph to be a chemolithoheterotroph. The apparent $Y_{sulphide}$ and $Y_{thiosulphate}$ were comparable, being $8-10$ g dry weight/mol. In batch cultures thiosulphate concentrations up to 10 mM had a stimulatory effect on the growth rate of *Hyphomicrobium* EG, whereas higher concentrations increased the organisms doubling time.

Enzyme- and respiration data showed that the organism had constitutive enzymes for the breakdown of dimethyl sulphoxide although they were clearly regulated to need. Addition of sulphide or thiosulphate to methylamine-limited chemostat cultures of *Hyphomicrobium* EG not only resulted in the induction of enzymes necessary for their breakdown, but also caused the enzymes for dimethyl sulphoxide metabolism, especially methyl mercaptan oxidase, to be induced. The formation of H_2O_2, a product of the latter enzyme, was reflected in the relatively high catalase activities during growth on dimethyl sulphoxide and in the organisms inability to grow on this compound in the presence of a catalase inhibitor.

Key words: *Hyphomicrobium* EG — Chemolithoheterotrophy — Methylotrophy — Dimethyl sulphoxide — Dimethyl sulphide — Thiosulphate — Sulphide — Yields — Physiology

In the past many heterotrophic bacteria have been shown to be able to oxidize reduced inorganic sulphur compounds (Starkey 1935; Trudinger 1967; Ruby et al. 1981; Lettl 1984). Only a few obtained metabolically useful energy from this oxidation and could therefore be considered true chemolithoheterotrophs (Rittenberg 1969; Kelly and Kuenen 1984). Among these are a number of marine pseudomonads (Tuttle et al. 1974; Tuttle 1980) and *Thiobacillus* Q (Gommers, personal communication). Recently the chemolithoheterotrophic status of *Thiobacillus perometabolis* (London and Rittenberg 1967) has been questioned by Katayama-Fujimura and Kuraishi (1983) who reported that this organism could be grown autotrophically.

In a recent study of the microbial breakdown of methylated sulphur compounds we isolated *Hyphomicrobium* EG which could grow well on dimethyl sulphoxide (DMSO) and dimethyl sulphide (DMS) (Suylen and Kuenen 1986). This organism proved to be an obligate, serine pathway utilizing methylotroph, which could also grow on methylated amines but not on methanol. During growth on DMS(O) it converted the sulphur part of this compound stoicheiometrically to sulphate. This raised the question whether the oxidation of sulphide would yield metabolically useful energy to this organism, in which case this would be the first demonstration of the existence of a chemolithoheterotrophic methylotroph. The only other methylotrophs possessing chemolithotrophic abilities are a *Hyphomicrobium* sp. which can grow autotrophically on hydrogen (Uebayasi et al. 1981, 1985), and *Paracoccus denitrificans* and *Thiobacillus versutus* which grow autotrophically on methanol (Cox and Quayle 1975; Kelly and Wood 1982) or reduced sulphur compounds (Friedrich and Mitrenga 1981; Gottschal and Kuenen 1980a). Recently Kanagawa and Kelly (1986) described a *Thiobacillus thioparus* strain which could grow autotrophically on DMS.

This paper reports on the methylotrophic and chemolithotrophic properties of *Hyphomicrobium* EG as observed in batch and chemostat cultures on DMSO, DMS and mixtures of methylamine and sulphide or thiosulphate.

Materials and methods

Organisms. *Hyphomicrobium* EG, deposited in the Delft culture collection under number LMD 84.101, was isolated as described earlier (Suylen and Kuenen 1986). *Hyphomicrobium* X and *Arthrobacter* P_1 (Levering et al. 1984) were obtained from the Delft culture collection numbers LMD 81.27 and 81.60 respectively.

Media and culture conditions. The basal medium for continuous cultivation of *Hyphomicrobium* EG contained (g l^{-1}): K_2HPO_4, 0.8; KH_2PO_4, 0.3; $MgSO_4 \cdot 7 H_2O$, 0.4; NH_4Cl,

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0.4; and 2.0 ml of a trace element solution as described by Vishniac and Santer (1957), except that it contained 2.2 g of $ZnSO_4 \cdot 7H_2O$ instead of the originally reported 22 g. Magnesium sulphate plus ammonium chloride and the trace element solution were sterilized separately. Different amounts of DMSO, methylamine and thiosulphate were added to this medium before sterilization; pure, undiluted DMS was added after the medium had been sterilized. NH_4Cl was omitted from methylamine containing media. The thiosulphate concentration in the medium feed of the culture was gradually increased from 1 via 3 to 5 mM, and above this concentration thiosulphate was added every three days in 0.6 mM amounts to a final concentration of 8.5 mM. Sulphide was fed separately to the chemostat culture; its concentration in the medium feed was increased from 1 to 5 mM and from thereon it was added in 0.5 mM portions every three days until a final concentration of 10 mM was reached. It was dissolved in deoxygenated demineralized water, and autoclaved like the other media for 20 min at 120°C. It was cooled under a gentle N_2 flow. During cultivation on sulphide a low N_2 flow was continually led over the solution as well as the drip through and the burette used for dilution rate (D) measurements. The dilution rate of DMSO- and DMS-limited chemostat cultures had to be taken up in small (0.003 h^{-1}) steps every 3−4 days to the desired dilution rate to prevent the cultures from washing out. A Biolafitte glass fermentor with a working volume of 1.5 l was used for the continuous cultivation. The pH was maintained at 7.0 by automatic titration with 1 N NaOH, the dissolved O_2 concentration was 50% of air saturation, the temperature 30°C and the D 0.035 h^{-1}. For continuous cultivation on DMS and sulphide the culturing system was equipped with butyl rubber or Teflon tubing and Tygon pump tubing. DMS containing medium was administered under the liquid level of the culture to prevent DMS-losses.

Hyphomicrobium EG and X and *Arthrobacter* P_1 were grown in batch culture either in Kluyver flasks or on a rotary shaker (225 rpm) on a medium which contained (g l^{-1}): $Na_2HPO_4 \cdot 7H_2O$, 7.9; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; $(NH_4)_2SO_4$, 0.4; and 2.0 ml of the trace element solution. The initial pH was 7.2. Different amounts of C_1-compounds and thiosulphate were added to this medium according to the description in the results section. When required, media were solidified by addition of 2% (w/v) Difco Bacto agar. Purity of the cultures was routinely checked by streaking on medium to which methylamine or DMSO and 0.05% (w/v) yeast extract had been added, and on a rich medium which contained (g l^{-1}): Difco tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0; the pH was adjusted to 7.2 and it was sterilized for 20 min at 120°C. No growth of the *Hyphomicrobium* species can occur on the latter medium. All chemicals used were of analytical grade.

Respiration measurements. Respiration rates of whole cells were measured polarographically with a Clark type oxygen electrode (Biological Oxygen Monitor, Yellow Springs Instrument Co., OH, USA) at 30°C on samples from chemostat cultures. The methyl mercaptan (CH_3SH, MM) dependent oxygen uptake rate was recorded by adding 50 μl of a freshly prepared MM solution with a concentration of approximately 3 mM. This was made by sparging deoxygenated, demineralized water with MM for 3−5 min. The obtained solution was a hundred times diluted with

deoxygenated water and stirred during 1 h for equilibration purposes. Rates are expressed in nmol O_2 min^{-1} (mg protein)$^{-1}$ and were corrected for endogenous respiration.

Analytical procedures. DMSO, DMS and MM were determined with a gaschromatograph as described earlier (Suylen and Kuenen 1986). MM concentration in MM solutions was measured spectrophotometrically using Ellman's reagent modified according to Kuwata et al. (1982). Methylamine was determined with amine oxidase from *Arthrobacter* P_1 (grown on ethylamine) as described by Levering et al. (1984). Sulphide and sulphite were determined according to Trüper and Schlegel (1964), thiosulphate according to Sörbo (1957), formaldehyde according to Houle et al. (1970), formate as described by Battat et al. (1974). Protein contents of whole cells and cell-free extracts were determined by the microbiuret method (Goa 1953), except that the cells were hydrolysed during 1 h 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. Total organic carbon in cell suspensions and culture supernatants was measured using a Beckman Tocamaster model 915-B.

Preparation of cell-free extracts. Cell-free extracts were made by disrupting cells by sonification as described earlier (Suylen and Kuenen 1986).

Enzyme assays. Ribulose-1,5-bisphosphate carboxylase (RubPCase) was determined by measuring $^{14}CO_2$ fixation as described by Beudeker et al. (1980). Phosphoenolpyruvate carboxylase (PEPCase) was determined as RubPCase. The reaction mixture contained: 100 mM Tris-HCl pH 8.2, 20 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM $NaHCO_3$, 5 mM PEP, 2.5 mM NADH, 0.4 mM acetyl CoA, 3 units of malate dehydrogenase and 1 mM $NaH^{14}CO_3$ (specific activity 1 μCi/μmol). Omission of malate dehydrogenase from the reaction mixture (the preparation contained NH_4^+-ions which could be inhibitory to the enzyme; Large et al. 1962) and/or addition of Mn^{2+}-ions (some PEPCases are Mn^{2+} rather than Mg^{2+} dependent; Sahl and Trüper 1977) did not affect the activity measurements. MM-oxidase activity was measured in fresh extracts in 50 mM Tris-HCl pH 8.2 with a Biological Oxygen Monitor. All other enzyme determinations were done spectrophotometrically (at their pH-optimum and at substrate concentrations at which maximal activity was observed) using a Hitachi model 100-60 with a temperature cuvette housing at 30°C. DMSO reductase was determined according to de Bont et al. (1981) at pH 8.0 with 0.6 mM DMSO. Hydroxypyruvate reductase was determined at pH 6.0 according to Large and Quayle (1963); NAD(P)$^+$-dependent formaldehyde dehydrogenase at pH 8.0 according to Stirling and Dalton (1978); 2,6-dichlorophenolindophenol (DCPIP)- and GSH-NAD(P)$^+$-dependent formaldehyde dehydrogenase at pH 7.5 according to Johnson and Quayle (1964) with 0.45 mM DCPIP, 0.3 mM phenazine ethosulphate (PES) and 200 and 3 mM formaldehyde respectively; N,N,N',N'-tetramethyl-p-phenylenediamine (Wurster's blue; TMPD)-dependent formaldehyde dehydrogenase as the DCPIP-dependent enzyme, only DCPIP and PES were replaced by 0.1 mM Wurster's blue; NAD(P)$^+$-dependent formate dehydrogenase at pH 7.0 according to Patel and Hoare (1971);

Table 1. Yield data on *Hyphomicrobium* EG. Dry weight and protein of *Hyphomicrobium* EG grown on different concentrations of various growth limiting substrates in a chemostat at $D = 0.035 \text{ h}^{-1}$. MA, methylamine; Y, yield

Concentration of growth limiting substrate	Dry weight (mg l^{-1})	Protein (mg l^{-1})	$Y_{mol\,C}$ (g dry weight/ mol carbon)
10 mM DMSO	167 ± 6	69 ± 2	8.35 ± 0.30
20 mM DMSO	330 ± 2	131 ± 5	8.25 ± 0.05
10 mM DMS	191 ± 4	79 ± 1	9.55 ± 0.20
10 mM MA	108 ± 3	47 ± 4	10.80 ± 0.30
20 mM MA	213 ± 4	77 ± 3	10.65 ± 0.20

methylamine oxidase and methylamine dehydrogenase according to Eady and Large (1968); N-methylglutamate dehydrogenase at pH 7.5 according to Bamforth and Large (1977a); catalase at pH 7.0 according to Lück (1963); NAD$^+$- and NADP$^+$-dependent malate dehydrogenase at pH 9.0 and 5.5 respectively according to Ochoa (1955); NAD(P)$^+$-dependent isocitrate dehydrogenase at pH 8.0 according to Ochoa (1948); isocitrate lyase at pH 7.0 according to Dixon and Kornberg (1959); L-serine glyoxylate aminotransferase at pH 8.0 according to Harder and Quayle (1971). PEPCase activities are expressed in nmol bicarbonate fixed min^{-1} (mg protein)$^{-1}$; MM oxidase activities in nmol $O_2 \text{ min}^{-1}$ (mg protein)$^{-1}$; catalase activities as $\Delta E_{240} \text{ min}^{-1}$ (mg protein)$^{-1}$; the other activities are given in nmol pyridine nucleotide (DCPIP, Wurster's blue or phenyl hydrazine) oxidized or reduced min^{-1} (mg protein)$^{-1}$. Isocitrate lyase activities smaller than 2 nmol phenyl hydrazone formed min^{-1} (mg protein)$^{-1}$ were considered insignificant and were probably due to interference of isocitrate dehydrogenase activity (Quayle 1975; Attwood and Harder 1977). Whenever it was judged necessary (as was e.g. the case with the PEPCase activities) cell-free extracts from differently grown cells were combined to check for the presence of stimulatory or inhibitory factors.

Results

Comparison of yields of Hyphomicrobium EG on DMSO, DMS and methylamine

Table 1 shows that *Hyphomicrobium* EG was growing under carbon and energy limitation in the chemostat: doubling the methylamine or DMSO concentration in the medium feed to the culture resulted in a doubling of its cell- and protein yield. No intermediates of the respective metabolic pathways were detectable in the culture supernatants. *Hyphomicrobium* EG was grown at approximately 50% air saturation on methylamine since it had been found that at oxygen tensions higher than about 65% air saturation considerably lower yields were obtained and the organisms μ_{max} decreased. In the steady states no excretion products were detectable, not even at the higher inhibitory oxygen tensions.

The $Y_{methylamine}$ was much lower than that on either DMSO or DMS. From a comparison of the metabolic pathways of methylamine and DMSO (Fig. 1) it is clear that these compounds are energetically equivalent as far as the metabolism of the methyl groups is concerned. Possible differences in DMSO and methylamine uptake requirements

Table 2. Yield data on *Hyphomicrobium* EG. Dry weight, protein and total organic carbon of *Hyphomicrobium* EG grown mixotrophically on methylamine (MA) and increasing concentrations of thio(sulphate) or sulphide (S^{2-}) at $D = 0.035 \text{ h}^{-1}$. All cultures were MA limited. In the MA plus sulphide cultures sulphide was not detectable

Substrate	Dry weight (mg l^{-1})	Protein (mg l^{-1})	Total organic carbon (mg l^{-1})	Residual thio in culture (mM)
10 mM MA	108 ± 3	47 ± 4	51 ± 2	0
10 mM MA + 1.1 mM thio	116 ± 6	42 ± 4	69 ± 1	0.26 ± 0.04
10 mM MA + 5.5 mM thio	144 ± 1	56 ± 3	72 ± 1	1.10 ± 0.04
10 mM MA + 8.55 mM thio	165 ± 3	68 ± 2	85 ± 1	1.65 ± 0.05
9.8 mM MA + 0.93 mM S^{2-}	116 ± 7	46 ± 6	56 ± 1	0
9.8 mM MA + 5.4 mM S^{2-}	152 ± 3	55 ± 1	73 ± 1	0.08 ± 0.04
9.5 mM MA + 10.4 mM S^{2-}	212 ± 3	77 ± 2	96 ± 1	0.11 ± 0.05

Table 3. Rates of substrate dependent oxygen uptake by *Hyphomicrobium* EG. Respiration rates (expressed in nmol $O_2 \text{ min}^{-1}$ [mg protein]$^{-1}$) of *Hyphomicrobium* EG grown on 10 mM DMSO at various D's (expressed in h^{-1}) and on 10 mM DMS at $D = 0.033 \text{ h}^{-1}$ after a 6 weeks (short) and 6 months (long) cultivation period on DMS. n. t. = not tested

Substrate	DMSO			DMS	
	$D = 0.01$	$D = 0.02$	$D = 0.035$	short	long
100 µM DMSO	472	529	758	406	392
100 µM DMS	527	640	798	526	1224
30 µM MM	728	377	281	n.t.	1088
30 µM Na$_2$S	544	435	452	482	814
50 µM sulphite	0	0	0	0	0
5 mM thiosulphate	92	136	163	107	144
1 mM formaldehyde	70	65	168	125	123
3 mM formate	37	41	45	40	51
1–10 mM methylamine	n.t.	n.t.	0	n.t.	0
10 mM TMAO	n.t.	n.t.	n.t.	n.t.	33

and energy obtained in the first step in methylamine metabolism, which in this organism is by way of formation of N-methylglutamate, are neglected. The latter compound is subsequently oxidized to formaldehyde with a NAD$^+$-independent N-methylglutamate dehydrogenase (see also Table 5). The difference in yield on DMS(O) and methylamine therefore might very well be caused by energy obtained from the oxidation of the sulphur part of these compounds, assuming the formaldehyde dehydrogenases under both growth conditions to be the same (see also Table 5). The following experiments were designed to test this possibility.
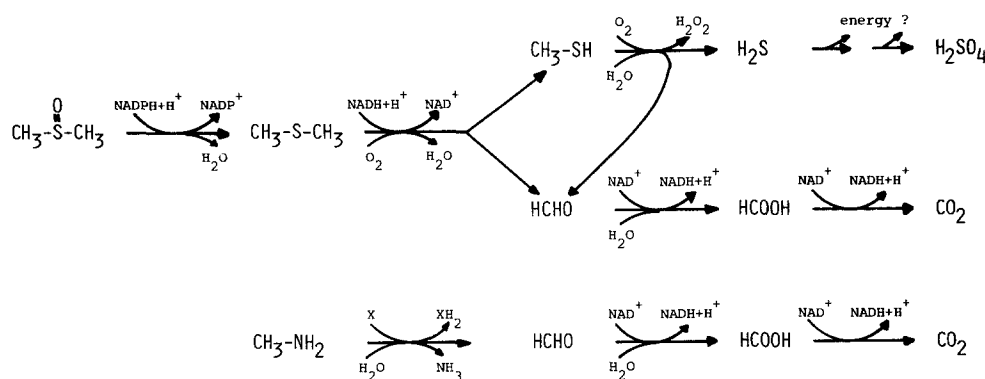
Fig. 1
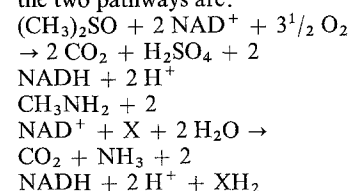Pathway of DMSO and methylamine metabolism. The net reaction equations of the two pathways are:

$(CH_3)_2SO + 2\,NAD^+ + 3^1/_2\,O_2$
$\rightarrow 2\,CO_2 + H_2SO_4 + 2$
$NADH + 2\,H^+$
$CH_3NH_2 + 2$
$NAD^+ + X + 2\,H_2O \rightarrow$
$CO_2 + NH_3 + 2$
$NADH + 2\,H^+ + XH_2$

Table 4. Rates of substrate dependent oxygen uptake by *Hyphomicrobium* EG. Respiration rates (expressed in nmol O_2 min^{-1} [mg protein]$^{-1}$) of *Hyphomicrobium* EG grown on 10 mM methylamine (MA) and increasing concentrations of thiosulphate or sulphide at $D = 0.035\,h^{-1}$. n.t. = not tested

Substrate	10 mM MA + thiosulphate				10 mM MA + sulphide		
	0 mM	1.0 mM	5.1 mM	8.5 mM	1.0 mM	5.4 mM	10.4 mM
100 μM DMSO	24	32	42	59	39	72	0
100 μM DMS	41	59	59	79	55	120	0
50 μM MM	52	122	95	209	132	200	745
40 μM Na₂S	119	174	118	189	291	298	692
50 μM sulphite	0	0	0	n.t.	0	0	n.t.
5 mM thiosulphate	0	13	47	76	24	73	134
4 mM formaldehyde	122	166	122	143	170	183	150
4 mM formate	32	39	24	37	45	56	39
1 mM methylamine	65	120	67	88	104	100	69

Comparison of Hyphomicrobium EG and X regarding their capacity for thiosulphate utilization in batch culture

When *Hyphomicrobium* EG was grown in batch culture on 10 mM methylamine with varying concentrations of thiosulphate (0–25 mM), it used 0.5–2.5 mM of the added thiosulphate, the amount used being independent on the initial concentration. In contrast *Hyphomicrobium* X, an organism incapable of growth on methylated sulphur compounds, used none of the added thiosulphate when grown under similar conditions, indicating that thiosulphate oxidation is not a common feature among hyphomicrobia. Variation of the methylamine concentration (2.5–10 mM) in medium with a fixed thiosulphate concentration (20 mM) did not have an effect on the thiosulphate utilization by *Hyphomicrobium* EG. Analogously, after growth on 10 mM dimethylamine or 10 mM trimethylamine N-oxide (TMAO) with 20 mM thiosulphate the organism used only slightly more thiosulphate (3.0–4.0 mM), so there was no correlation between the concentration of methyl groups in the medium and the amount of thiosulphate utilized. In all cases only a slight decrease in pH (about 0.2 units) was observed. Upon reaching the stationary phase which coincided with C_1-compound depletion the cultures continued to utilize thiosulphate. This oxidation was not coupled to biomass formation, indicating that the organism was unable to grow autotrophically. Thiosulphate had a concentration dependent effect on the maximum specific growth rate of *Hyphomicrobium* EG on methylamine (pregrown in a

DMSO-limited chemostat): 5–10 mM caused an increase from 0.10 to 0.11 h^{-1}, 15–20 mM a decrease to 0.075 h^{-1}. Yields of batch cultures of *Hyphomicrobium* EG on 10 mM methylamine plus a variable amount of thiosulphate ranged from 135 to 143 mg dry weight l^{-1} and were slightly higher than those on 10 mM methylamine alone (130–136 mg dry weight l^{-1}) and the final E_{430} of the cultures differed by 15%. Although this suggested that some energy was gained by thiosulphate oxidation this was not considered conclusive evidence. Therefore chemostat studies in which thiosulphate or sulphide were added to a methylamine-limited *Hyphomicrobium* EG culture were undertaken.

Yields of Hyphomicrobium EG on mixtures of methylamine and thiosulphate or sulphide

The yield data in Table 2 show that *Hyphomicrobium* EG is able to derive energy from the oxidation of thiosulphate and sulphide during growth in a methylamine-limited chemostat. The methylamine plus thiosulphate grown *Hyphomicrobium* EG cultures did not become thiosulphate limited (Table 2): thiosulphate accumulated in the culture with increasing thiosulphate concentrations in the medium feed, indicating that the organism had a low affinity for this compound. Thiosulphate was also detectable (0.34 mM) in a chemostat culture grown on 10 mM DMSO plus 2.91 mM thiosulphate. The methylamine plus sulphide grown *Hyphomicrobium* EG cultures also accumulated small

Table 5. Enzyme activities in cell-free extracts of *Hyphomicrobium* EG. Enzyme activities of *Hyphomicrobium* EG grown on 10 mM DMSO at different D's (expressed in h^{-1}), on 10 mM DMS at $D = 0.033\ h^{-1}$ and on 10 mM methylamine (MA) in the presence and absence of 5.1 mM thiosulphate (T) or 10.4 mM sulphide (S) at $D = 0.035\ h^{-1}$. Enzyme activities are expressed in nmol min^{-1} (mg protein)$^{-1}$ except for the catalase activity which is given as $\Delta E_{240}\ min^{-1}$ (mg protein)$^{-1}$. n.t. = not tested; red. = reductase; deh. = dehydrogenase; a.t. = aminotransferase

Enzyme	Coenzyme	DMSO			DMS	MA	MA + 5.1 T	MA + 10.4 S
		$D = 0.01$	$D = 0.02$	$D = 0.035$				
DMSO red.	NADPH	9	40	107	16	1	1	35
	NADH	18	26	31	6	9	6	8
MM oxidase		163	70	56	268	67	104	379
Catalase		3.72	2.94	3.57	7.56	0.61	1.01	1.26
Formaldehyde deh.	NAD$^+$	33	33	29	36	61	69	69
	TMPD	726	563	364	302	1106	1148	1273
Formate deh.	NAD$^+$	20	67	146	115	128	154	177
Serine glyoxylate a.t.	NADH	n.t.	n.t.	476	300	453	496	395
Hydroxypyruvate red.	NADH	5056	5444	5246	6863	4534	3816	4397
	NADPH	49	66	53	44	53	n.t.	n.t.
PEPCase		n.t.	n.t.	76	19	87	219	60
RubPCase		n.t.	n.t.	0	0	0	0	0
Malate deh.	NADPH	106	94	200	281	405	n.t.	n.t.
	NADH	1807	1337	1217	1530	1601	n.t.	n.t.
Isocitrate deh.	NADP$^+$	32	28	54	75	96	n.t.	n.t.
N-Methyl glutamate deh.	DCPIP	n.t.	n.t.	0	0	15	12	11
	NAD(P)$^+$	n.t.	n.t.	n.t.	n.t.	0	n.t.	n.t.
Methylamine deh.	NAD(P)$^+$	n.t.	n.t.	n.t.	n.t.	0	n.t.	n.t.
Methylamine oxidase		n.t.	n.t.	n.t.	n.t.	0	n.t.	n.t.

amounts of thiosulphate, whereas sulphide was not detectable ($< 0.5\ \mu M$). This might suggest that some chemical oxidation of the sulphide had taken place (Chen et al. 1972).

Respiration- and enzyme data on Hyphomicrobium EG grown under various conditions

The enzyme activities and respiration rates of *Hyphomicrobium* EG measured after growth on different substrates (Tables 3 – 5) gave additional support for the metabolic pathway of DMS(O) as proposed by de Bont et al. (1981). In most cases there was a good correlation between respiration- and enzyme data, the trends being an increase in both activities with increasing D's (except for MM oxidation; Tables 3 and 5) and induction of enzymes of the DMS(O) metabolic pathway upon addition of thiosulphate/ sulphide to methylamine-limited chemostat cultures (Tables 4 and 5).

The effect of the growth rate of *Hyphomicrobium* EG was studied during growth on DMSO (Tables 3 and 5). The NAD$^+$-dependent formaldehyde dehydrogenase activity did not vary with the growth rate (Table 5), whereas formaldehyde respiration did (Table 3), suggesting another enzyme to be involved in formaldehyde oxidation. No NADP$^+$-dependent formaldehyde dehydrogenase activity was detectable and GSH, known to be involved in the formaldehyde oxidation by some other organisms (Anthony 1982) had no effect on either of these enzymes. A mixture of PES and DCPIP (DCPIP on its own gave 15% of the activity found with the mixture) or Wurster's blue could be used as electron acceptors (Wurster's blue being thrice as effective as the mixture). The enzyme had a low affinity for formaldehyde (apparent K_m 170 mM) and acetaldehyde (apparent K_m 12 mM). Butyl aldehyde and formate were

also substrates, so the enzyme could also be a general aldehyde dehydrogenase. Although the formate dehydrogenase activity (Table 5) was high enough to explain the organisms growth rate, the respiration of externally added formate was comparatively slow (Table 3). This may well be due to the fact that only the free acid passes the cell membrane: respiration rates found at pH 6.2 and 5.6 were, respectively, 47 and 94% higher than those at pH 7.2. The NADPH necessary for the functioning of DMSO reductase (Table 5) might be supplied by the NADP$^+$-dependent isocitrate- and malate dehydrogenases.

The effect of different substrates and auxiliary energy sources was studied at a constant growth rate to ensure comparability of the results (Tables 4 and 5). The respiration- and enzyme data of *Hyphomicrobium* EG, a serine pathway utilizing methylotroph, grown on 10 mM DMSO plus 2.9 mM thiosulphate at $D = 0.035\ h^{-1}$ (not shown in the Tables) were comparable to the results obtained with the DMSO only grown cells, except for the activity of PEPCase, a key enzyme of the serine pathway, which was 245 nmol bicarbonate fixed min^{-1} (mg protein)$^{-1}$. The changes in the observed PEPCase activity (Table 5) could not be explained: no stimulatory factor was present in the cell-free extract of cells grown in the presence of thiosulphate (see also Materials and methods). Furthermore, the PEPCase activity in different extracts of similarly grown cells varied considerably. The isocitrate lyase activity found under the various growth conditions was insignificant (see also Materials and methods), as was expected since none of the other known *Hyphomicrobium* spp. possess significant isocitrate lyase activity (Attwood and Harder 1977).

The catalase activity found in DMSO or DMS grown cells was 5 – 12 times higher than that in methylamine grown cells, which stressed the enzymes role in the removal of H_2O_2
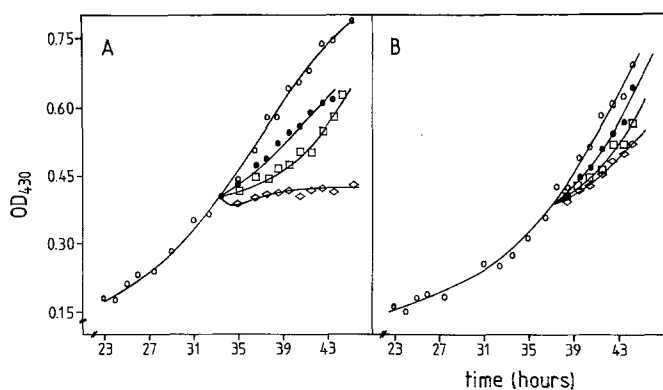
Fig. 2A, B. Effect of addition of (●) 20, (□) 30 and (◇) 50 mM 3-amino 1,2,4-triazole (AT) to *Hyphomicrobium* EG cultures in the exponential growth phase; (○) nothing added. *Hyphomicrobium* EG was grown on **(A)** 10 mM DMSO or **(B)** 10 mM methylamine. AT was added after **(A)** $33^1/_2$ and **(B)** $37^1/_2$ h

formed during methyl mercaptan oxidation. The effect of 3-amino-1,2,4-triazole, a catalase inhibitor (Cohen and Somerson 1969), on the growth of *Hyphomicrobium* EG on DMSO (Fig. 2), further demonstrated the indispensability of H_2O_2 removal during growth on DMSO.

Although whole cells were unable to respire sulphite (Table 3) cell-free extracts did (data not shown) indicating the possibility of sulphite being an intermediate in the oxidation of sulphide to sulphate. The organisms ability to oxidize sulphide was clearly constitutive. Thiosulphate was only oxidized by cells which had been cultured on a medium which contained an inorganic or organic reduced sulphur compound (Tables 3 and 4), suggesting its formation as a by-product of chemical and/or biological sulphide oxidation (see also Table 2).

Discussion

The presented data show that substrate limited chemostat cultivation is indispensable in establishing the chemolithoheterotrophic behaviour of *Hyphomicrobium* EG. In contrast, batch cultures in which all compounds are abundant proved unsuitable for the demonstration of reproducable yield increases from sulphide or thiosulphate oxidation. Our failure to obtain conclusive evidence for chemolithoheterotrophic growth in batch cultures is in accordance with observations with sulphur oxidizing chemolithotrophs (Kelly and Kuenen 1984; Gommers, personal communication). DMSO metabolism by *Hyphomicrobium* EG is via reduction to DMS (Table 5), which explains the higher yield on the latter compound (19.1 g dry weight/mol) as compared to that on DMSO (16.7 g dry weight/mol). The Y_{DMSO} is higher again than that on methylamine (10.8 g dry weight/mol), which suggested at first sight that the organism gains energy from the oxidation of the sulphur moiety of DMSO (see also Fig. 1). This indeed proved to be the case in subsequent studies on *Hyphomicrobium* EG cultures grown on mixtures of methylamine and sulphide or thiosulphate (Table 2). The apparent $Y_{thiosulphate}$ and $Y_{sulphide}$ which can be calculated from the data in Table 2 by subtracting the dry weight value found on methylamine from that on methylamine plus thiosulphate/sulphide and dividing the obtained value by the amount of thiosulphate/sulphide used by the culture, are

comparable and around $8-10$ g dry weight/mol. The so derived yields are in accordance with those found for mixotrophic thiobacilli (Perez and Matin 1980; Gottschal and Kuenen 1980a, b).

The percentage of methylamine assimilated (carbon conversion efficiency) during growth of *Hyphomicrobium* EG on methylamine alone was 21 or 28% depending on the assumed formaldehyde/CO_2 fixation ratio in the serine pathway (1 or 2; Anthony 1982). It increased after growth on mixtures of 10 mM methylamine and 10.4 mM sulphide or 5.5 mM thiosulphate (to 42 or 56% and 30 or 40% respectively). This implies that even at much higher sulphide/thiosulphate concentrations in the medium feed autotrophic CO_2 fixation would not be required, hence the lack of RubPCase activity in this organism (Table 5).

The relatively low oxidative capacity (Tables 3 and 4) and high substrate affinity constant (K_s) for thiosulphate (around 0.5 mM; Suylen, unpublished data), also illustrated by the accumulation of substantial amounts of thiosulphate under the used growth conditions (Table 2), indicate that exogenously administered thiosulphate is not a good substrate. Although the situation is much more favourable with respect to sulphide, the K_s of *Hyphomicrobium* EG for this compound is still relatively high (values of 17 and 22 μM were found after growth on DMSO and methylamine respectively; Suylen, unpublished data) compared to that of the *Thiobacilli* which is around 1 μM. However, *Hyphomicrobium* EG may well have evolved as an organism specialized in the breakdown of methylated sulphur compounds since it has a high affinity for DMSO, DMS and MM (Suylen and Kuenen 1986; and unpublished data) also in comparison with the affinity for DMS found in *Thiobacillus thioparus* (Kanagawa and Kelly 1986). Its inability to grow autotrophically (Suylen and Kuenen 1986) also points to this.

The specialist behaviour of *Hyphomicrobium* EG was further indicated by the fact that the enzymes for DMS(O) metabolism are constitutive (Tables 4 and 5), in contrast with those for methylamine breakdown which need inducing (Tables 3 and 5) as was also found to be the case with other methylamine utilizers (Boulton and Large 1977; Bamforth and Large 1977a, b; Bamforth and O'Connor 1979). Furthermore, the fact that sulphide and thiosulphate, which both appear to be formed during the breakdown of DMS(O), are inducers for the enzymes of the complete metabolic pathway (Tables 4 and 5) also demonstrates the specialist character of *Hyphomicrobium* EG. Finally, the higher respiration rates of DMS and Na_2S found after growth for over 6 months on DMS relative to those found after a shorter cultivation period on it (Table 3), which are possibly due to a change in the organisms genetic configuration, indicate that *Hyphomicrobium* EG may specialize even further under the stress of circumstances.

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