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ORIGINAL PAPER

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Enzymes of dimethylsulfone metabolism and the phylogenetic characterization of the facultative methylotrophs *Arthrobacter sulfonivorans* sp. nov., *Arthrobacter methylotrophus* sp. nov., and *Hyphomicrobium sulfonivorans* sp. nov

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Abstract Novel methylotrophic *Arthrobacter* and *Hyphomicrobium* species are described. Constitutive membrane-associated dimethylsulfone- and dimethylsulfoxide-reductases were found in *Arthrobacter methylotrophus* strain TGA and *Hyphomicrobium sulfonivorans* strain S1. Enzyme activities increased during growth with dimethylsulfone or dimethylsulfoxide, respectively, and different ratios of activity with different growth substrates indicated that they are separate enzymes. SDS-PAGE showed some membrane-associated polypeptides to be enhanced during growth with dimethylsulfone (54 kDa in *H. sulfonivorans*, 21–24 kDa, 54 kDa and 80 kDa in *A. methylotrophus*). Western blotting with anti-dimethylsulfoxide-reductase antibody showed cross-reaction with 54- and 21-kDa polypeptides in *A. methylotrophus*. All strains contained rhodanese and sulfur oxygenase after growth with dimethylsulfone. Sulfite was oxidized in the *Arthrobacter* species by APS reductase and sulfite dehydrogenase. *H. sulfonivorans* oxidized sulfite with APS reductase, which is unusual for an α-proteobacterium. The *Arthrobacter* species were distinguished from each other and from other *Arthrobacter* and *Micrococcus* species by 16S rRNA gene sequence analysis. The menaquinone and fatty acid profiles of the *Arthrobacter* species were similar. Their peptidoglycan structures were L-Lys–L-Ser–L-Thr–L-Ala for *A. sulfonivorans* and L-Lys–L-$Ala_{2–4}$ for *A. methylotrophus*. *H. sulfonivorans* exhibited gross morphology typical for *Hyphomicrobium*, but possessed helically twisted prosthecae. 16S rRNA gene sequence analysis showed it to be distinct from all the other *Hyphomicrobium*, *Filomicrobium* and *Pedomicrobium* species sequenced to date. Formal descriptions of the new species are given.

Keywords *Arthrobacter sulfonivorans* · *Arthrobacter methylotrophus* · *Hyphomicrobium sulfonivorans* · Dimethylsulfone · Reductases · Regulation · Taxonomy · Sulfur oxidation

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Introduction

We previously described new methylotrophic bacteria, provisionally identified as novel species of *Arthrobacter* and *Hyphomicrobium* (Borodina et al. 2000). The significance of these organisms was twofold: (1) they were the first bacteria described in detail as able to degrade dimethylsulfone, which does not undergo chemical breakdown at a measurable rate and is not attacked by the most powerful acids, alkalis or oxidizing or reducing agents (Clarke 1911; Dean 1992); (2) they indicated a route for the biodegradation of atmospheric dimethylsulfone produced by the chemical oxidation of biogenic dimethylsulfide (Harvey and Lang 1986; Watts et al. 1990; Yin et al. 1990; Berresheim et al. 1993, 1998; Bandy et al. 1996; Kelly and Murrell 1999; Borodina et al. 2000).

Bacteria grown with dimethylsulfone also oxidize dimethylsulfoxide or dimethylsulfide, but the ability to use dimethylsulfone is specifically induced by dimethylsulfone. Dimethylsulfone degradation proceeds by its reduction by NADH-dependent reductases via dimethylsulfoxide to dimethylsulfide, followed by monooxygenation of the dimethylsulfide to produce methanethiol and formaldehyde: $(CH_3)_2SO_2 \rightarrow (CH_3)_2SO \rightarrow (CH_3)_2S \rightarrow CH_3SH + HCHO$.

The methanethiol is oxidized to formaldehyde and sulfide: $CH_3SH + 0.5O_2 \rightarrow HCHO + H_2S$ and the sulfide oxidized to sulfate: $H_2S + 2O_2 \rightarrow H_2SO_4$.

Part of the formaldehyde is oxidized to carbon dioxide and the remainder assimilated as the primary carbon source for growth either by the serine pathway or the ribulose monophosphate cycle (Borodina et al. 2000).

There have been few detailed studies of the biochemistry of *Arthrobacter* strains able to grow methylotrophically (Loginova and Trotsenko 1976; Levering et al. 1981a, b, 1984), and no *Hyphomicrobium* strain had previously been described as able to use dimethylsulfone as a sole source of carbon and energy for growth, although some other strains have been shown to use dimethylsulfoxide (De Bont et al. 1981; Suylen and Kuenen 1986; Suylen 1988). Morphological and physiological characteristics of strain S1 have been described previously (Borodina et al. 2000).

We now show that dimethylsulfone and dimethylsulfoxide appear to be reduced by separate reductase enzyme activities, that there is modulation of these activities by growth substrate, and that enzymes for inorganic sulfur oxidation leading to sulfate formation are present in all three strains, but sulfite oxidation differs in the *Hyphomicrobium* from that in the *Arthrobacter* species.

The two *Arthrobacter* strains are formally described as distinct species within the genus, based on their 16S rRNA gene sequences, menaquinones, fatty acid profiles and peptidoglycan composition. The *Hyphomicrobium* strain is shown to exhibit unusual hyphal morphology and is formally described as *Hyphomicrobium sulfonivorans* sp. nov.

Materials and methods

Bacterial strains, morphological and biochemical properties

Hyphomicrobium strain S1, *Arthrobacter* strain TGA, and *Arthrobacter* strain ALL were isolated, maintained and cultured as described previously (Borodina et al. 2000).

Methods for biochemical characterization, SDS-PAGE, DNA isolation and determination of base composition, the sequencing of 16S rRNA genes, and calculation of their similarity values, evolutionary distances and generation of phylogenetic dendrograms were as described by Borodina et al. (2000). Cell-free extracts for enzyme assays were prepared by passing cell suspensions in 55 mM phosphate, pH 8.0, three times through a French pressure cell at 110 MPa. Broken cell preparations were centrifuged at 35,000×*g* for 30 min at 4 °C and the supernates used as a cell-free extract. For preparation of membrane and cytoplasmic fractions, broken cell preparations were centrifuged at 12,100×*g* to remove unbroken cells and large debris, and were then centrifuged at 150,000×*g* for 1.5 h. The supernatant liquid was used as the cytoplasmic fraction (which included the periplasmic material in the case of *Hyphomicrobium*) and the pellet was used as the membrane fraction.

Pairwise identity comparisons of the 16S rRNA gene sequences of the three strains with public database sequences of related bacteria used the BLAST2 method of Tatusova and Madden (1999; http://www.ncbi.nlm.nih.gov/gorf/bl2.html). Samples of *Hyphomicrobium* strain S1 were prepared for electron microscopy by drying on formvar-coated grids, and were then negatively stained with ammonium molybdate or platinum-shadowed. Samples were examined using a JEOL 100CX MKII transmission electron microscope. Dimethylsulfone and dimethylsulfoxide reductases were assayed as described previously (Borodina et al. 2000). Activities of sulfur oxygenase, sulfite oxidase, adenylylsulfate (APS) reductase, rhodanese, and tetrathionate synthase were assayed according to Kelly and Wood (1994, 1998).

Immunological visualization of dimethylsulfoxide reductase

Immunoblotting for dimethylsulfoxide reductase in subcellular fractions of *Hyphomicrobium* strain S1 and *Arthrobacter* strain TGA was carried out with the affinity-purified anti-dimethylsulfoxide-reductase (*Rhodobacter capsulatus*) polyclonal antibody, described by Hatton et al. (1994). After SDS-PAGE, polypeptides on the gels were electroblotted on to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, UK) using a Mini Trans-Blot Cell (Bio-Rad, UK) run at 100 V for 1 h at 4 °C. Membranes, after blocking, were incubated with anti-dimethylsulfoxide-reductase antibody (Hatton et al. 1994), washed, incubated with Bio-Rad alkaline phosphatase (AP) conjugate (goat anti-rabbit IgG AP; Bio-Rad, UK), washed, developed with 50 µl Bio-Rad enhancer reagent in 10 ml Bio-Rad Immun-Star chemiluminescent substrate (Bio-Rad, UK), and the membranes exposed to Hyperfilm MP X-ray film (Amersham International, UK) for 5–7 min.

Analysis of peptidoglycans, fatty acids and menaquinones of the *Arthrobacter* strains

Procedures used for these determinations were as described by Groth et al. (1999). Peptidoglycan types are described according to the key given by DSMZ (2000; http://www.dsmz.de/species/murein.htm), and the terminology used to describe the isoprenoid quinones of the *Arthrobacter* strains is that used by Collins and Jones (1981).

Accession numbers

The GenBank accession numbers for the 16S rRNA sequences of strains S1, TGA and ALL are AF235089, AF235090 and AF235091, respectively.

Results and discussion

Dimethylsulfone reductase and dimethylsulfoxide reductase are distinct enzymes in *Hyphomicrobium* strain S1 and *Arthrobacter* strain TGA

High activities of these reductases were found in both strains grown with dimethylsulfone or dimethylsulfoxide (Table 1). High activities were also seen in bacteria grown with other one-carbon compounds, indicating constitutive synthesis.

In *Hyphomicrobium* strain S1, the highest activity of dimethylsulfone reductase was found after growth with dimethylsulfone, while dimethylsulfoxide reductase was most active in bacteria grown with dimethylsulfoxide (Table 1). Student's *t* test was applied to 6–12 separate activity val-

Table 1 Activities of dimethylsulfone and dimethylsulfoxide reductases in subcellular fractions of *Hyphomicrobium sulfonivorans* strain S1 and *Arthrobacter methylotrophus* strain TGA grown on different one-carbon substrates. Reductase activities are expressed as nmol NADH oxidized min^{-1} (mg protein)$^{-1}$. Values are the means (± standard deviation for 4–12 determinations). Assays of malate dehydrogenase as a marker cytoplasmic enzyme in all assays showed minimal contamination between the fractions (Borodina et al. 2000)

Growth substrate (mM)	Dimethylsulfone reductase		Dimethylsulfoxide reductase	
	Membrane fraction	Cytoplasmic fraction	Membrane fraction	Cytoplasmic fraction
Hyphomicrobium sulfonivorans				
Dimethylsulfone (10,20)	70±12	5±1	49± 8	2±1
Dimethylsulfoxide (2)	57± 5	0	118±11	0
Methanol (10)	49± 3	0	72± 4	0
Formate (20)	35± 6	0	55± 3	0
Arthrobacter methylotrophus				
Dimethylsulfone (10,20)	37± 8	0	41±11	2±1
Dimethylsulfoxide (2)	35± 3	0	86± 8	0
Methylamine (10)	16± 2	0	69± 9	0

ues to estimate the significance of the differences shown in Table 1. The difference between dimethylsulfone reductase and dimethylsulfoxide reductase in *Hyphomicrobium* strain S1 grown with dimethylsulfone was highly significant at the 0.1% level. The difference for bacteria grown with dimethylsulfoxide was also highly significant at the 0.1% level. Similarly, the difference observed between dimethylsulfone reductase activity in *Hyphomicrobium* strain S1 grown with dimethylsulfone or dimethylsulfoxide was significant at the 1% level; and the difference between dimethylsulfoxide reductase activity in bacteria grown with dimethylsulfone or dimethylsulfoxide was highly significant at the 0.1% level.

For *Arthrobacter* strain TGA there was no significant difference between the dimethylsulfone reductase and dimethylsulfoxide reductase activities in bacteria grown with dimethylsulfone or between the dimethylsulfone reductase activities of bacteria grown with either dimethylsulfone or dimethylsulfoxide (Table 1). The difference between the dimethylsulfoxide reductase activities in bacteria grown with dimethylsulfone or dimethylsulfoxide (Table 1) was, however, highly significant at the 0.1% level in the *t* test.

Dimethylsulfoxide reductase activity in *Hyphomicrobium* strain S1 grown with dimethylsulfoxide or methanol increased to levels 1.5 to 2.5 times greater than those found in bacteria grown with dimethylsulfone, while dimethylsulfone reductase activity was significantly lower after growth with dimethylsulfoxide or methanol (Table 1). Similarly, dimethylsulfoxide reductase activity relative to dimethylsulfone reductase activity was much greater in *Arthrobacter* strain TGA grown on dimethylsulfoxide or methylamine than on dimethylsulfone (Table 1). The specific activity of dimethylsulfone reductase in methylamine-grown bacteria was half the value seen in dimethylsulfone-grown organisms, while the dimethylsulfoxide reductase activity was almost double that seen after growth with dimethylsulfone (Table 1). This variation in the relative activity of the two reductases suggested that each reaction was catalyzed by a distinct enzyme protein. Ratios of dimethylsulfoxide reductase activity/dimethylsulfone reductase activity were determined from several different cultures, for each of which the reproducibility of enzyme activities was better than ±10%. For *Hyphomicrobium* strain S1, the ratios of activities after growth with dimethylsulfone or dimethylsulfoxide were about 0.7 and 2.1, and about 1.5 after growth on methanol or formate. The ratio was about 1.0 for *Arthrobacter* strain TGA grown with dimethylsulfone but 2.5 after growth with dimethylsulfoxide. After growth of *Arthrobacter* strain TGA with methylamine the ratio of activities was 4.3.

While dimethylsulfone reductase activity was constitutive in *Hyphomicrobium* strain S1 grown with methanol and *Arthrobacter* strain TGA grown with methylamine, transfer into medium with dimethylsulfone resulted in a doubling of activity in both organisms (Table 2). After an initial decrease, activity of dimethylsulfoxide reductase also increased to a level above that of the methanol-grown inoculum in *Hyphomicrobium* strain S1 (Table 2). The initial high activity of dimethylsulfoxide reductase in methylamine-grown *Arthrobacter* strain TGA decreased about 30% during subsequent growth with dimethylsulfone, to the level typically seen with strain TGA grown with dimethylsulfone (Tables 1 and 2).

At least 95% of both enzyme activities was always recovered in the membrane fraction of the two strains (Table 1; Borodina et al. 2000), and the low activities occasionally seen in the cytoplasmic fraction (which included the periplasm in the case of *Hyphomicrobium* strain S1) were attributed to minor contamination by membrane material. Dialysis of the cytoplasmic fractions from both strains for 18 h at 4 °C against 50 mM phosphate buffer, pH 6.8, hardly affected the cytoplasmic activity of either enzyme, suggesting that they were absent rather than masked by a diffusible autoinhibitor.

SDS-PAGE and Western blotting of membrane-fraction polypeptides

Membrane fractions from *Hyphomicrobium* strain S1 grown with dimethylsulfone or dimethylsulfoxide and *Arthrobacter* strain TGA grown with dimethylsulfone were subjected to SDS-PAGE, electroblotting, and reac-

Table 2 Changes in activities of dimethylsulfone (*$DMSO_2$*) and dimethylsulfoxide (*DMSO*) reductases in *Hyphomicrobium sulfonivorans* strain S1 and *Arthrobacter methylotrophus* strain TGA grown on 10 mM methanol or 10 mM methylamine, then exposed to 20 mM dimethylsulfone for 5–28 h. Reductase activities are expressed as nmol NADH oxidized min^{-1} (mg protein)$^{-1}$ and are the sum of those found in the membrane and cytoplasmic fractions (typically 95–100% of activity was in the membrane fraction)

Growth conditions (time)	$DMSO_2$ reductase	DMSO reductase
Hyphomicrobium sulfonivorans		
Dimethylsulfone (20 mM, 60 h) from a dimethylsulfone-grown inoculum	85±4	51±2
Methanol-grown inoculum	49±3	72±4
Dimethylsulfone (5 h)	57±1	47±3
Dimethylsulfone (16 h)	68±1	59±4
Dimethylsulfone (28 h)	111±9	92±8
Arthrobacter methylotrophus		
Dimethylsulfone (20 mM, 60 h) from an inoculum grown on dimethylsulfone	32±2	44±4
Methylamine-grown inoculum	16±2	69±9
Dimethylsulfone (5 h)	33±3	64±9
Dimethylsulfone (16 h)	36±4	56±8
Dimethylsulfone (28 h)	43±4	49±4

tion with anti-dimethylsulfoxide-reductase polyclonal antibody as described in Materials and methods. The results obtained with the antibody were treated with caution as strains S1 and TGA are aerobes using dimethylsulfone and dimethylsulfoxide as growth substrates whereas earlier work, including that with the *Rhodobacter* antibody, has been with bacteria using these compounds as oxidants for anaerobic respiration. The 85-kDa periplasmic dimethylsulfoxide reductase of *Hyphomicrobium* EG did, however, show immunological similarity to the 80-kDa periplasmic enzyme from *Rhodobacter* (Hatton et al. 1994), suggesting that this approach was potentially useful.

Hyphomicrobium strain S1

Comparison of SDS-PAGE polypeptide profiles of the membrane fractions from strain S1 grown either with dimethylsulfone or methanol showed only one major difference: a polypeptide of about 54 kDa (Fig. 1). The 54-kDa polypeptide increased in intensity during exposure of growing cultures to dimethylsulfone but was most intense in a 60-h culture (Fig. 1A, B). The 54-kDa polypeptide was also strongly expressed in the membrane fractions of *Hyphomicrobium* strain S1 grown with dimethylsulfone in continuous chemostat culture (Fig. 1B). The finding that

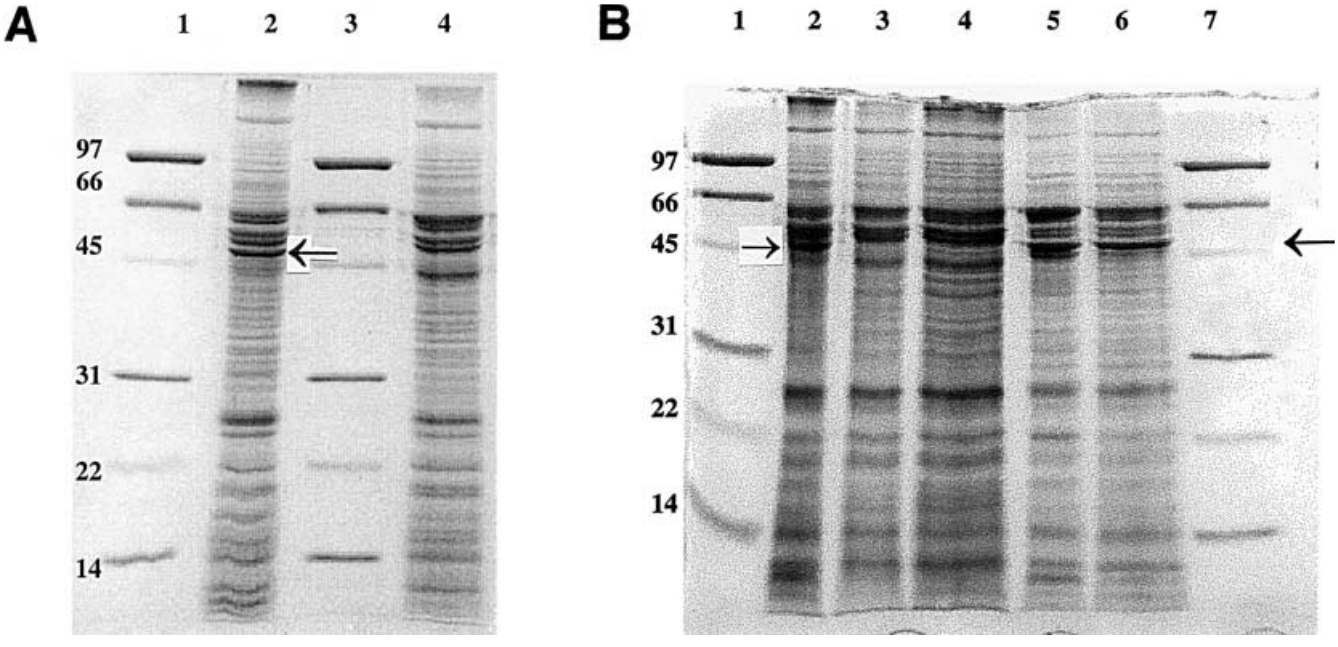

Fig. 1A, B Polypeptide profiles of the membrane fraction of *Hyphomicrobium sulfonivorans.* **A** After 60-h growth with 20 mM dimethysulfone (*lane 2*) or 10 mM methanol (*lane 4*); *lanes 1, 3* protein standards with the molecular masses (in kDa) indicated. The *arrow* indicates the 54-kDa polypeptide seen in *lane 2*. **B** After growth with 20 mM dimethylsulfone (*lane 2*), or pre-growth with 10 mM methanol followed by growth with 20 mM dimethylsulfone for 5 h (*lane 3*) or 28 h (*lane 4*); or growth in a dimethylsulfone-limited continuous-flow chemostat at dilution rates of 0.08 h^{-1} (*lane 5*) or 0.15 h^{-1} (*lane 6*); *lanes 1, 7* molecular mass marker proteins. The *arrow* indicates the position of the 54-kDa polypeptide (see text)

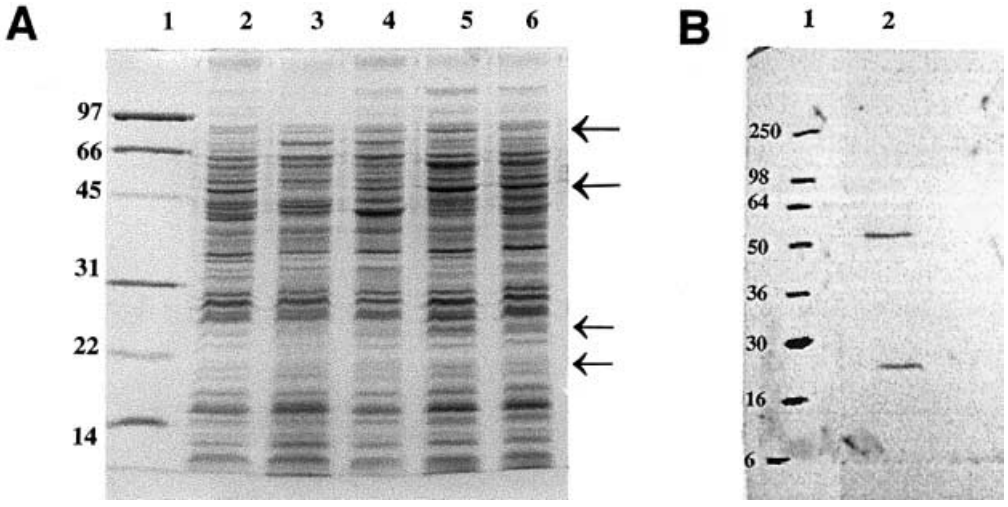

Fig. 2 **A** Polypeptide profiles of the membrane fraction of *Arthrobacter methylotrophus* after 60-h growth with 20 mM dimethysulfone (*lane 2*) or 10 mM methylamine (*lane 3*); or after pre-growth with 10 mM methylamine followed by growth with 20 mM dimethylsulfone for 5 h (*lane 4*), 16 h (*lane 5*) or 28 h (*lane 6*); *lane 1* protein standards with the molecular masses (in kDa) indicated. The *arrows* indicate the position of the 80-kDa, 54-kDa and 21–24-kDa polypeptides (see text). **B** Membrane fraction of *Arthrobacter methylotrophus* after 60-h growth with 20 mM dimethysulfone, immunoblotted using anti-dimethylsulfoxide reductase antibody (*lane 2*); *lane 1* molecular mass marker proteins. The bands in *lane 2* have molecular masses of about 54 kDa and 21 kDa

the 54-kDa polypeptide was most intense in dimethylsulfone-depleted cultures (i.e. in chemostat cultures and the 60-h batch culture), and was only weakly expressed in some extracts showing high dimethylsulfone- and dimethylsulfoxide-reductase activities (Fig. 1; Table 2), suggests it might be a transport or binding protein involved in dimethylsulfone uptake, highly expressed only at low dimethylsulfone concentrations.

Immunoblotting with anti-dimethylsulfoxide-reductase antibody of the gel from strain S1 grown with dimethylsulfone revealed only very faint cross-reacting polypeptides of about 60, 35 and 23 kDa, but no reaction with the 54-kDa polypeptide. As the dimethylsulfoxide reductase enzymes from *Rhodobacter* and *Hyphomicrobium* EG are monomers of molecular mass of about 80 or 85 kDa (Hatton et al. 1994; Knablein et al. 1997), and the membrane-bound respiratory dimethylsulfoxide reductase from *Escherichia coli* is a trimer with subunits of 82.6, 23.6 and 22.7 kDa (Weiner et al. 1988), only the 23 kDa-polypeptide might have corresponded with a subunit from the other reductases.

Arthrobacter strain TGA

Comparison of SDS-PAGE polypeptide profiles of the membrane fractions from strain TGA grown with dimethylsulfone or methylamine showed high expression of a 54-kDa polypeptide only after growth with dimethylsulfone (Fig. 2A). Progressive increases were also seen during growth with dimethylsulfone in a fainter polypeptide band of about 80 kDa and several polypeptide bands of 21–24 kDa (Fig. 2A, lanes 4–6).

Anti-dimethylsulfoxide-reductase antibody reacted with two polypeptides of about 54 and 21 kDa in the membrane fraction (Fig. 2B). The cross-reacting 21-kDa polypeptide was absent from the cytoplasmic fraction and only a weak reaction was seen with a polypeptide of about 54 kDa in the cytoplasmic fraction. While reaction with two polypeptides may be an artifact, as the antibody was raised against a monomeric enzyme, the possibility cannot be excluded that the 21-kDa polypeptide is a fragment of the 54-kDa polypeptide or that both are fragments of a larger molecule, both containing regions reacting with the antibody. The polypeptides of 21, 54 and about 80 kDa in *Arthrobacter* strain TGA may thus be components of dimethylsulfone and dimethylsulfoxide reductases.

The induction of a 54-kDa polypeptide in both the *Hyphomicrobium* and *Arthrobacter* strains grown with dimethylsulfone suggests this may be a specific common component of dimethylsulfone degradation, but we have not yet been able to determine whether it is a component of the reductase or functions in dimethylsulfone binding.

Enzymes of dissimilatory sulfur compound metabolism in *Hyphomicrobium* strain S1 and *Arthrobacter* strains TGA and ALL

All three strains grew with dimethylsulfide and dimethylsulfoxide as well as on dimethylsulfone, and enzymes catalyzing the formation of formaldehyde and hydrogen sulfide are present in all three strains (Borodina et al. 2000). *Hyphomicrobium* strain S1 also oxidized inorganic sulfide, and all three strains produced sulfuric acid during growth with dimethylsulfone (Borodina et al. 2000). Enzymes likely to be involved in the oxidation of the sulfide produced from methanethiol were demonstrated in all three strains (Table 3). The high activities of rhodanese and sulfur oxygenase found in all three suggested that the oxidation of the sulfide-sulfur of methanethiol may in-

Table 3 Enzymes of inorganic sulfur oxidation in cell-free extracts of the *Hyphomicrobium* and *Arthrobacter* strains. Activities were assayed as: oxygen uptake for sulfur oxygenase; ferricyanide reduction for APS reductase, sulfite dehydrogenase and tetrathionate synthase; thiocyanate formation for rhodanese. Numbers in *brackets* indicate the number of assays performed. *nd* Not determined

Enzyme	Activity [nmol min^{-1} (mg protein)$^{-1}$]		
	Hyphomicrobium strain S1	*Arthrobacter* strain TGA	*Arthrobacter* strain ALL
Sulfur oxygenase	535±258 [3]	113± 8 [2]	296±99 [2]
APS reductase	59± 11 [3]	28±11 [3]	16 [1]
Sulfite dehydrogenase	0 [31]	13± 5 [2]	12 [1]
Rhodanese	163± 35 [3]	152±34 [3]	309±12 [2]
Tetrathionate synthase	0	0	nd

volve a sulfurtransferase reaction and direct dioxygenation of sulfur to produce sulfite (Kelly 1999). In *Hyphomicrobium* strain S1 the oxidation of sulfite to sulfate appears to be catalyzed only by an AMP-dependent APS reductase but in the *Arthrobacter* strains both AMP-dependent and AMP-independent sulfite oxidation was shown (Table 3). Since it is likely that sulfur for biosynthesis will be derived from the sulfide arising from sulfone reduction, the APS reductase probably functions exclusively for sulfite oxidation to sulfate. For the *Hyphomicrobium* strain, this would be the first example of this enzyme catalyzing sulfite oxidation in a member of the α-Proteobacteria (Friedrich 1998; Kappler et al. 2001). The possible contribution of sulfide oxidation to energy metabolism in the three strains has not yet been quantified (Kelly 1999).

Taxonomic characterization of the *Arthrobacter* strains and *Hyphomicrobium* strains

Methylotrophy is relatively uncommon in *Arthrobacter* species and dimethylsulfone metabolism by *Hyphomicrobium* is also unusual, with other strains tested previously being unable to use it. Consequently we deemed it essential to carry out a more detailed taxonomic analysis of all three strains to establish their relatedness to other species within their respective genera.

DNA base ratios and phylogenetic analysis of *Arthrobacter* strains TGA and ALL

The DNA of both *Arthrobacter* strain ALL and strain TGA were found to contain 61 mol% G+C.

A phylogenetic tree for nearly complete sequences of the 16S rRNA genes of strains TGA and ALL (Fig. 3) confirmed that they differed at the species level both from each other and from the reference species of *Arthrobacter* and *Micrococcus*. Strain ALL clustered loosely on the dendrogram with *A. oxydans* and *A. polychromogenes*, but strain TGA appeared more distantly related to *A. globiformis*, *A. pascens* and *A. ramosus* (see also Borodina et al. 2000). Comparison of the identities (%) of the 16S rRNA gene sequence of strains ALL and TGA with each other and with other *Arthrobacter* species was analyzed using the BLAST2 method (Tatusova and Madden 1999). This showed strains ALL and TGA to share 97.3% identity. Strain ALL shared 96.8–97.7% identity with *A. oxydans*, *A. polychromogenes*, *A. pascens*, *A. ramosus*, *A. woluwensis*, *A. globiformis*, *A. nicotinivorans*, *A. psychrolactophilus*, *A. aurescens* and *A. nicotianae*; and 95.0–96.7% identity with *A. luteolus* (AJ243422), *A. albus* (AJ243421), *A. protophormiae*, *A. ureafaciens*, *A. cumminsii*, *A. sulfureus* and *A. rhombi*. Strain TGA showed 96.8–97% identity to *A. pascens*, *A. ramosus*, *A. aurescens*, *A. woluwensis* and *A. globiformis*; and 94.8–96.5% identity to all the other species listed. Stackebrandt and Goebel (1994) reported that, in general, if the sequence similarity between two strains is below 97.5%, the DNA-DNA similarity between the strains is unlikely to be more than 60–70%. Consequently, strains sharing less than 97.5% sequence identity are unlikely to be related at the species level (Stackebrandt and Goebel 1994). As strains ALL and TGA share only 97.3% identity with each other, and less than about 97% identity to other species, they are unlikely to be related at the species level either to each other or to other *Arthrobacter* species. For comparison, *A. pascens* showed 100% identity to *A. ramosus*, and 99% identity was seen between *A. oxydans* and *A. polychromogenes*, and between *A. globiformis* and *A. pascens*.

Cellular fatty acid composition, quinone content, peptidoglycan composition and structure of *Arthrobacter* strains TGA and ALL

Both *Arthrobacter* strains contained 13-methylpentadecanoic acid (anteiso-C15:0) as the dominant cellular fatty acid (Table 4). This fatty acid is typically predominant in all the *Arthrobacter* species studied to date (Keddie et al. 1986; Crocker et al. 2000). Significant quantities of 14-methylpentadecanoic acid (iso-C15:0), 15-methylhexadecanoic acid (iso-C16:0), and 15-methylheptadecanoic acid (anteiso-C17:0) were also present in both strains (Table 4), as is common in environmental isolates and numerous established species of *Arthrobacter* (Crocker et al. 2000).

Menaquinone MK-9(H_2) comprised about 80% of the total menaquinone content of both strains. The proportions of MK-9(H_2): MK-10(H_2): MK-8(H_2): MK-9: MK-7(H_2): MK-11(H_2) in each strain were: strain TGA, 79: 7: 6: 4: 2: 1; strain ALL, 80:8:5:2:1:1.

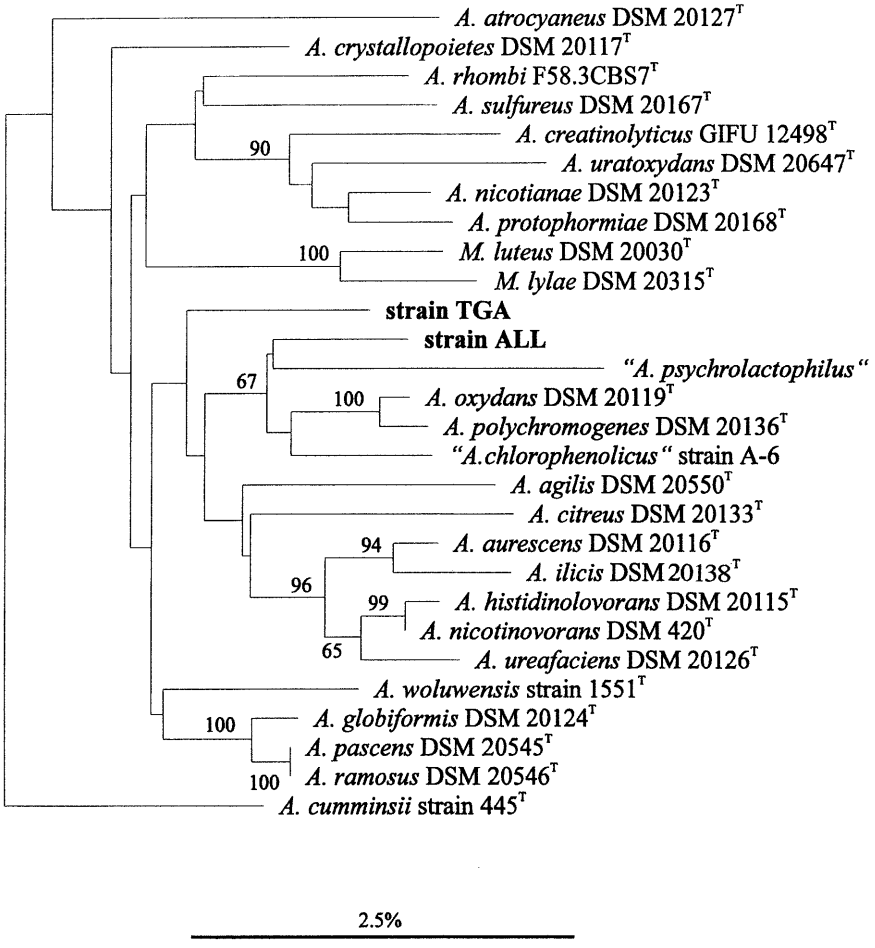

Fig. 3 Phylogenetic tree based on 16S rRNA gene sequence analysis of members of the genus *Arthrobacter* and *Micrococcus*, showing the position of *Arthrobacter* strains TGA and ALL. The accession numbers of the reference sequences used in the phylogenetic analysis are as follows: strain TGA, AF235090; strain ALL, AF235091; *A. agilis*, X80748; *A. atrocyaneus*, X80746; *A. aurescens*, X83405; "*A. chlorophenolicus*", AF102267; *A. citreus*, X80737; *A. creatinolyticus* (JCM 10102^T; Hou et al. 1998), D88211; *A. crystallopoietes*, X80738; *A. cumminsii*, X93354; *A. globiformis*, X80736; *A. histidinolovorans*, X83406; *A. ilicis*, X83407; *Micrococcus lylae*, X80750 (Koch et al. 1994); *Micrococcus luteus*, M38242; *Arthrobacter nicotianae*, X80739; *A. nicotinovorans*, X80743; *A. oxydans*, X83408; *A. pascens*, X80740; *A. polychromogenes*, X80741; *A. protophormiae*, X80745; "*A. psychrolactophilus*" (ATCC 700733; Loveland-Curtze et al. 1999), AF134179; *A. ramosus*, X80742; *A. rhombi* (CCUG 38813^T; Osorio et al. 1999), Y15884; *A. sulfureus*, X83409; *A. uratoxydans*, X83410; *A. ureafaciens*, X80744; *A. woluwensis*, X93353. *CCUG* Culture Collection University of Göteborg, Sweden; *GIFU* Department of Microbiology, Gifu University, Japan. *Bar* 2.5 inferred nucleotide substitutions per 100 nucleotides

Two-dimensional thin-layer chromatography showed the peptidoglycan of both strains to contain lysine as the diagnostic diamino acid, as well as alanine and glutamic acid. Strain ALL also contained threonine and serine. Strain TGA contained a relatively high amount of alanine. From the major amino acids and the occurrence of characteristic peptides in partial hydrolysates of the peptidoglycans, provisional peptidoglycan structures were deduced. For strain TGA the data (L-Lys–L-Ala$_{2,\ 3\ \text{or}\ 4}$) indicated an A11.5, A11.6 or A11.7 structure, which is seen in *A. globiformis*, *A. pascens* and *A. ramosus*. Strain ALL contained the A11.23 peptidoglycan type (L-Lys–L-Ser–L-Thr–L-Ala), which is characteristic of *A. oxydans* and *A. polychromogenes*. These data are consistent with the phylogenetic tree (Fig. 3) and confirm that strains ALL and TGA are novel species of *Arthrobacter*.

Phylogenetic characterization of *Hyphomicrobium* strain S1

Inclusion of strain S1 in the *Hyphomicrobium* grouping was indicated by its characteristic morphology and 16S rRNA gene sequence analysis (Borodina et al. 2000). It was apparently not a member of either of the two clusters of species identified by Rainey et al. (1998) and seemed to represent the sole member of a third cluster within *Hyphomicrobium*. Comparison of its 16S rRNA gene sequence identity with that of other species of *Hyphomicrobium* and *Pedomicrobium* was assessed using the BLAST2 method (Tatusova and Madden 1999). The sequence showed only 92.9–95.3% identity to those of *H. vulgare* (Y14303 and X53182), *H. zavarzinii* (Y14305), *H. facile* (Y14309) and *H. methylovorum* (Y14307),

Table 4 Fatty acid profiles of *Arthobacter* strains TGA and ALL

Fatty acid determined	Fatty acid content (% of total lipids detected)	
	Arthrobacter strain TGA	*Arthrobacter* strain ALL
C14:0	0.9	1.4
C15:0	0.2	–
C16:0	1.6	3.9
C16:1	–	1.3
C18:1	0.7	–
i-C14:0	1.9	1.2
i-C15:0	7.1	15.2
i-C16:0	5.7	5.8
i-C17:0	0.3	1.3
ai-C15:0	74.7	62.5
ai-C17:0	6.8	7.1
ai-C17:1	–	0.4
Total	99.9	100.1

Pedomicrobium (Filomicrobium) fusiforme (DSM 5304; Y14313), *P. ferrugineum* (X97690), *P. manganicum* (X97691), *P. americanum* (X97692), and *P. australicum* (X97693 and X97694). These data indicate that strain S1 is not closely related to other *Hyphomicrobium* species, but from its morphological and phylogenetic properties is best described as a new species of that genus.

Twisted hyphal morphology of *Hyphomicrobium* strain S1

Electron micrographs of *Hyphomicrobium* strain S1 showed characteristic twisted prosthecae (Fig. 4). Morphology closely matching that seen with strain S1 was observed previously in a few other *Hyphomicrobium* isolates (Monosov and Kudinova 1976; Holm et al. 1996). Monosov and Kudinova (1976) reported that the distance between the turns of the helix in the prostheca of *Hyphomicrobium* strain M-1 was approximately the same as the diameter of the prostheca (0.2–0.3 μm), as is also seen in strain S1 (Fig. 4A). Morphologically, strain S1 is indistinguishable with respect to hyphal twisting and rosette formation from published electron micrographs of *Hyphomicrobium* strain IFAM 1465 (Fig. 4B; Gliesche et al. 1996; Holm et al. 1996). In a study of bacteriophage specific for hyphomicrobia, Gliesche et al. (1988) found four bacteriophages which attacked *Hyphomicrobium* strains with twisted hyphae but did not attack any of 40 other *Hyphomicrobium* strains or strains of *Pedomicrobium* (Holm et al. 1996). This is consistent with a closer relationship between strain S1 and the isolates of Gliesche et al. (1988, 1996; Holm et al. 1996) and Monosov and Kudinova (1976) than with other hyphomicrobia. Unfortunately 16S rRNA gene sequences are not available for strains M-1 and IFAM 1465, so phylogenetic similarity cannot yet be compared to determine if they and strain S1 all represent strains or species of a third cluster of *Hyphomicrobium*.

Description of *Arthrobacter methylotrophus* sp. nov.

Arthrobacter methylotrophus (me.thy.lo.tro'phus. M.L. [from Gr.] prefix *methylo-* pertaining to the [chemical] methyl group, Gr. v. *trephein* to feed, M.L. adj. *methylotrophus* feeding on methyl groups).

Cells are spherical or rod-shaped, showing a rod-coccus growth cycle, 0.6 μm in diameter and 1.3 μm in length. Gram-positive, forming clumps and chains in liquid culture, and are non-motile; spores or capsules not seen; catalase-and oxidase-positive. Grows heterotrophically and aerobically on glucose, fructose, sucrose, galactose, acetate, ethanol, pyruvate, malate, succinate, citrate, serine, alanine, taurine and yeast extract, and on alkanesulfonates (propane-, butane-, pentane- and hexane-sulfonate) and diethylsulfone. Grows aerobically on methylated sulfur compounds (dimethylsulfone, dimethylsulfoxide, dimethylsulfide), methanol, methylamine, trimethylamine and formaldehyde. Methylotrophic growth with dimethylsulfone uses the ribulose monophosphate cycle for formaldehyde assimilation. Methylotrophic growth with methanol uses the serine pathway for C_1-assimilation. Does not grow with methanesulfonate or autotrophically on inorganic sulfur compounds. Nitrate is not reduced. Growth on dimethylsulfone occurs optimally at pH 7.2–7.5 and at 25 °C. Temperature range for growth is 4–30 °C with no growth at 37 or 44 °C. Ammonium chloride, nitrate, methylamine and EDTA are used as nitrogen sources for growth. Growth occurs in the presence of 1.5% (w/v) NaCl, weakly with 2.5% (w/v) NaCl, but not with 5% (w/v) NaCl. On dimethylsulfone-agar medium they produce creamy-yellow, circular-convex colonies, 1.0–1.3 mm in diameter. MK-9(H_2) is the principal isoprenoid quinone with smaller amounts of MK-10(H_2), MK-8(H_2), MK-9, MK-7(H_2) and MK11(H_2). The principal cellular fatty acid is 13-methylpentadecanoic acid, with 14-methylpentadecanoic acid, 15-methylhexadecanoic acid and 15-methylheptadecanoic acid also present. Peptidoglycan contains lysine as the diagnostic diamino acid, as well as alanine and glutamic acid; provisional peptidoglycan type (L-Lys–L-Ala_{2-4}) is an A11.5, A11.6 or A11.7 structure. The G+C content of the DNA is 61 mol%. As determined by 16S rRNA gene sequence analysis the organism is closely related to other members of the genus. The type strain is strain TGA, which was isolated from soil from the root system of *Tagetes minuta*, and is deposited at the American Type Culture Collection under the accession number ATCC BAA-111, and at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as DSM 14008. The GenBank accession number for its 16S rRNA gene sequence is AF235090.

Description of *Arthrobacter sulfonivorans* sp. nov.

Arthrobacter sulfonivorans (sul.fo.ni.vo'rans. M.L. prefix *sulfoni* from the sulfone group, L. v. *vorare* devour, M.L. adj. *sulfonivorans* sulfone-devouring).

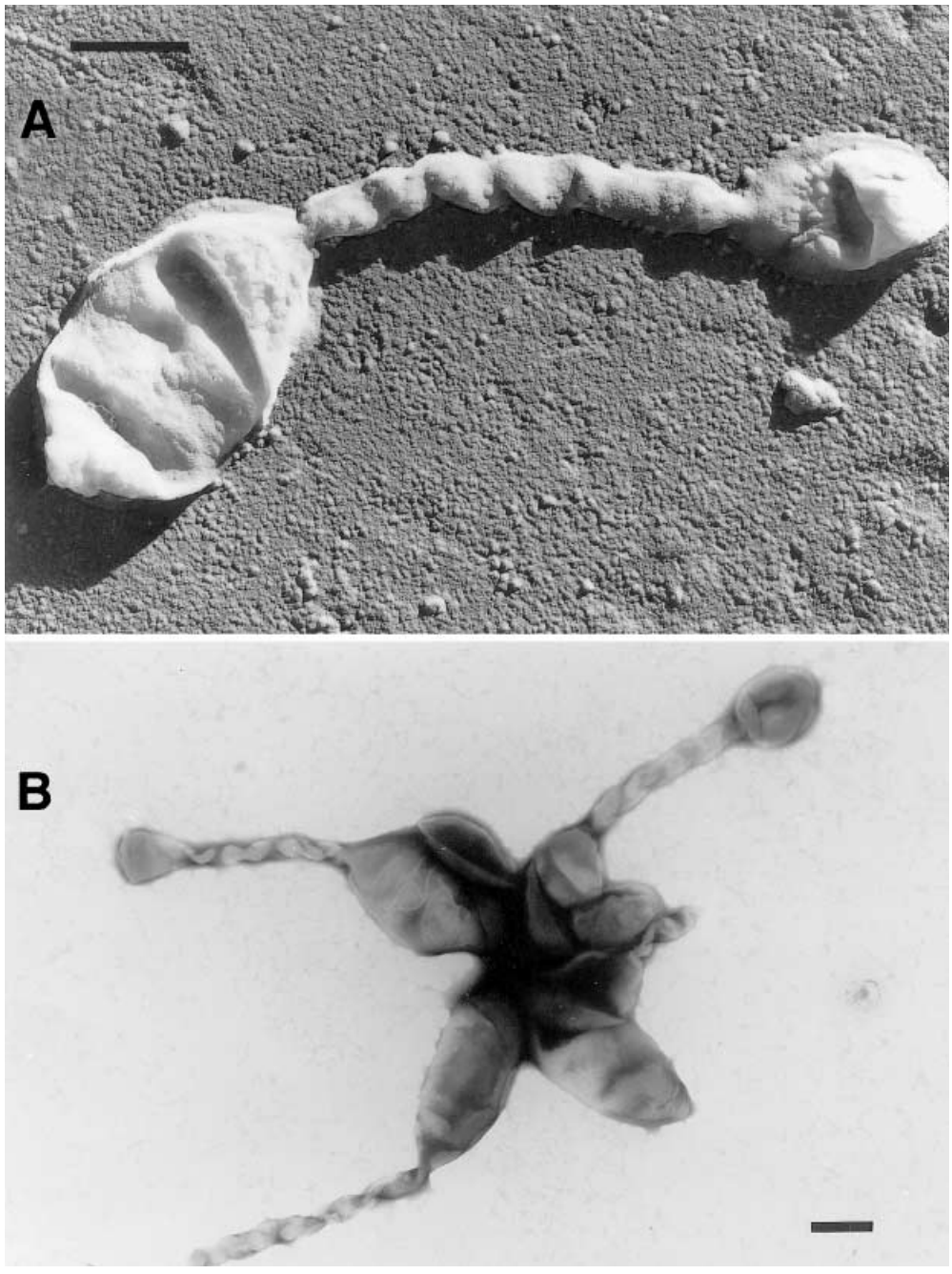

Fig. 4 Electron micrographs of *Hyphomicrobium sulfonivorans* strain S1, showing helically twisted prosthecae and rosette formation. **A** Single cell and prostheca (shadowed with platinum); **B** a rosette of four cells, also showing twisted prosthecae with terminal spores (negatively stained with ammonium molybdate). *Bar* 0.5 μm

Cells are spherical, 0.7 μm in diameter. Gram-positive, forming clumps and chains in liquid culture; motile; spores or capsules not seen; catalase- and oxidase-positive. Grows heterotrophically and aerobically on glucose, fructose, sucrose, galactose, acetate, ethanol, pyruvate, malate, succinate, citrate, serine, alanine, taurine, diethylsulfone, and yeast extract. Grows aerobically on methylated sulfur compounds (dimethylsulfone, dimethylsulfoxide, dimethylsulfide), methanol, methylamine and dimethylamine. Methylotrophic growth with dimethylsulfone uses the ribulose monophosphate cycle for formaldehyde assimilation. Does not grow with trimethylamine or alkanesulfonates (methane-, propane-, butane-, pentane- and hexane-sulfonate), or autotrophically on inorganic sulfur compounds. Nitrate is not reduced. Growth on dimethylsulfone occurs optimally at pH 7.3–7.4 and at 20–25 °C. Temperature range for growth is 4–30 °C with no growth at 37 or 44 °C. Ammonium chloride, nitrate and methylamine are used as nitrogen sources for growth. Growth occurs in the presence of 1.5% or 2.5% (w/v) NaCl, but not with 5% (w/v) NaCl. On dimethylsulfone-agar medium, produces creamy-yellow, circular-umbonate colonies, 0.5–0.7 mm in diameter. The principal isoprenoid quinone is MK-9(H_2), with smaller amounts of MK-10(H_2), MK-8(H_2), MK-9, MK-7(H_2) and MK11(H_2). The principal cellular fatty acid is 13-methylpentadecanoic

acid, with 14-methylpentadecanoic acid, 15-methylhexadecanoic acid and 15-methylheptadecanoic acid also present. Peptidoglycan contains lysine as the diagnostic diamino acid, as well as alanine, glutamic acid, threonine and serine; peptidoglycan type (L-Lys–L-Ser–L-Thr–L-Ala) is A11.23. The G+C content of the DNA is 61 mol%. As determined by 16S rRNA gene sequence analysis the organism is closely related to other members of the genus. The type strain is strain ALL, which was isolated from soil from the root ball of *Allium aflatunense* and is deposited at the American Type Culture Collection under the accession number ATCC BAA-112, and at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as DSM 14002. The GenBank accession number for its 16S rRNA gene sequence is AF235091.

Description of *Hyphomicrobium sulfonivorans* sp. nov.

Hyphomicrobium sulfonivorans (sul.fo.ni.vo'rans. M.L. prefix. *sulfoni* from the sulfone group, L. v. *vorare* devour, M.L. adj. *sulfonivorans* sulfone-devouring).

Cells are bean-shaped with a single prostheca, with budding at the ends of the prosthecae; 0.5 μm in width and 3.3 μm in length (including the prostheca). The prosthecae are about 2.3 μm in length and exhibit helical twisting. Gram-negative, forming clumps, rosettes and chains, with wall-growth and pellicles in liquid culture; motility variable; spores or capsules not seen; catalase- and oxidase-positive. Grows heterotrophically and aerobically on fructose, acetate, ethanol, pyruvate, malate, succinate, alanine, taurine, diethylsulfone, propane-, butane- and hexane-sulfonates, and on yeast extract. Grows aerobically on methylated sulfur compounds (dimethylsulfone, dimethylsulfoxide, dimethylsulfide, methanethiol), methanol, methylamine, trimethylamine, formaldehyde and formate. Methylotrophic growth with dimethylsulfone, methanol or formaldehyde uses the serine pathway for C_1-assimilation. Does not grow with glucose, galactose, lactate, citrate, dimethylamine, or methane- or pentane-sulfonate, or autotrophically on inorganic sulfur compounds. No anaerobic growth with denitrification on dimethylsulfone or pyruvate. Growth on dimethylsulfone occurs optimally at pH 7.3–7.6 and at 30 °C. Temperature range for growth is 15–37 °C with no growth at 4 °C or 44 °C. Ammonium chloride and methylamine are used as nitrogen sources for growth. Growth occurs in the presence of 1.5% (w/v) NaCl, but not with 2.5 or 5% (w/v) NaCl. On dimethylsulfone-agar medium, produces creamy-brown, circular-convex colonies, 0.2–0.3 mm in diameter. The G+C content of the DNA is 62 mol%. As determined by 16S rRNA gene sequence analysis the organism is not closely related to the other two phylogenetic clusters comprising this genus, but may be a third phylogenetic cluster within *Hyphomicrobium*. The type strain is strain S1, which was isolated from Warwickshire garden soil and is deposited at the American Type Culture Collection under the accession number ATCC BAA-113, and at the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, as DSM 13863. The GenBank accession number for its 16S rRNA gene sequence is AF235089.

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