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MINI-REVIEW

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Microbial metabolism of methanesulfonic acid

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Abstract Methanesulfonic acid is a very stable strong acid and a key intermediate in the biogeochemical cycling of sulfur. It is formed in megatonne quantities in the atmosphere from the chemical oxidation of atmospheric dimethyl sulfide (most of which is of biogenic origin) and deposited on the Earth in rain and snow, and by dry deposition. Methanesulfonate is used by diverse aerobic bacteria as a source of sulfur for growth, but is not known to be used by anaerobes either as a sulfur source, a fermentation substrate, an electron acceptor, or as a methanogenic substrate. Some specialized methylotrophs (including *Methylosulfonomonas, Marinosulfonomonas,* and strains of *Hyphomicrobium* and *Methylobacterium*) can use it as a carbon and energy substrate to support growth. Methanesulfonate oxidation is initiated by cleavage catalysed by methanesulfonate monooxygenase, the properties and molecular biology of which are discussed.

Key words Methanesulfonate · Oxidation · Reduction · Oxygenase · Energy · Methylotrophy

Introduction

While researching material for a review (Kelly and Smith 1990) more than a decade ago, one of us wrote to a colleague: "I have found that there is a sulfur compound that seems to have no 'metabolic history' of any consequence – methanesulfonic acid. This arises from the destruction of dimethyl sulfide in the atmosphere (about 20 million tonnes a year – not an insignificant amount) – where does it go?" (Extract from an unpublished letter from D. P. Kelly to A. P. Wood, University of Western Australia, 22 October 1988). Apart from very limited mention of methanesulfonic acid as a sulfur source for some organisms, no work had at that time been done to investigate the microbial degradation of this important intermediate in the global sulfur cycle. This was because the occurrence of methanesulfonate in the natural environment and its importance as an oxidation product of dimethyl sulfide were only recognized in recent years (Lovelock et al. 1972; Kelly and Murrell 1996; Ayers et al. 1997; Legrand 1997), and dimethyl sulfide itself was only fully established as the major atmospheric component of the sulfur cycle in the 1980s (Charlson et al. 1987; Murrell and Kelly 1996). Work over the past 20 years has shown conclusively that dimethyl sulfide, arising principally from the dimethylsulfoniopropionate [$(CH_3)_2S^+CH_2CH_2COO^-$] osmolyte of marine algae, is the major source of sulfur in the atmosphere, with an annual input of 40–70 million tonnes (Kelly 1996; Kelly and Murrell 1996; Kiene et al. 1996; Murrell et al. 1996; Lelieveld et al. 1997). In the atmosphere, dimethyl sulfide is oxidized in the light by OH radicals and in the dark by NO_3 radicals. Depending on the geographical latitude, 25–70% of the flux of dimethyl sulfide is oxidized to methanesulfonate, which is deposited back to the earth in rain, snow and by dry deposition (Kelly et al. 1993; Kelly and Murrell 1996; Murrell et al. 1996).

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Sulfonate chemistry and the special case of methanesulfonate

Sulfonic acids have the general formula RCH_2SO_3H, where R is H in the case of methanesulfonate, but can be a longer aliphatic chain or an aromatic ring, with or without additional substitutions. The common feature of sulfonates is their possession of a sulfur atom that is covalently linked to a carbon atom. The oxidation state of the sulfur in sulfonates has been reported both as +4 and +5 (Hanselmann 1991; Huxtable 1992; Seitz et al. 1993; Uria-Nickelsen at al. 1993a), but comparative spectrographic studies on a variety of compounds have shown that the electron distribution within the sulfonate ion results in the sulfur being at the +5 oxidation state (Frank et

al. 1987; Vairavamurthy et al. 1993; Kelly and Murrell 1996; Lie et al. 1998; Cook et al. 1999). Chemically, there are very great differences in the carbon-sulfur bond stability in the series of n-alkanesulfonic acids. Methanesulfonate is remarkably stable, while ethanesulfonate is decomposed relatively rapidly. The chemical decomposition rate of the longer chain alkanesulfonates decreases progressively as the carbon chain lengthens. Wagner and Reid (1931) measured hydrolysis rates (as release of sulfite) of aliphatic sulfonic acids in 3.7 M sodium hydroxide at 345 °C. Under these conditions only 1.5% of the sulfite was hydrolysed from methanesulfonate after 3 h, compared to 63% from ethanesulfonate, 20% from propanesulfonate and 12% from hexanesulfonate. Such data indicate that the absence of detectable methanesulfonate from soils and the marine environment is because of its biological rather than chemical degradation.

Methanesulfonate in climatic and palaeoecological microbiology contexts

Current climate models lay emphasis on the opposing phenomena of atmospheric warming as a result of changing concentrations of "greenhouse gases" (carbon dioxide, methane, etc.) and possible atmospheric cooling as a result of albedo modification brought about by changes in cloud formation. For the latter, cloud condensation nuclei (e.g. sulfate aerosols) are key triggers (Ayers et al. 1997; Jones and Slingo 1997), with methanesulfonate being part of the "atmospheric burden" of such nuclei (Ayers et al. 1997). The phenomenon of "negative cloud forcing", by which the atmosphere is cooled as a result of increased cloud formation, is clearly seen following catastrophic events such as the injection of massive amounts of sulfur dioxide into the atmosphere by major volcanic events (e.g. Mount Pinatubo in 1991; Hansen et al. 1997). Ongoing negative forcing due to aerosol effects on cloud formation is likely to explain why the global warming that has occurred progressively since the beginning of the European industrial revolution (i.e. for at least 200 years) is only about half of the predicted values (Hansen et al. 1997). On a geological timescale, analysis of ice cores from the Antarctic and Greenland gives a continuous measure of atmospheric methanesulfonate concentrations extending from recent times to over 10^5 years before the present (Legrand 1997). Methanesulfonate derived from atmospheric dimethyl sulfide must, however, have been an atmospheric component for much longer than 10^5 years, as coccolithophorids (dimethyl sulfide-producers) were superabundant in the oceans of the Cretaceous period (Robinson 1995). Methanesulfonate has thus had a continuous and significant input to marine and terrestrial environments (potentially at rates of about 50 Tg/year) for at least the past 100 million years (Kelly 1996). Ample time was thus available for the evolution of bacteria able to use methanesulfonate, and the absence of measurable quantities of methanesulfonate from soils and water indicates its ready degradation by the modern microflora (Kelly 1996).

Microbiological consumption of methanesulfonate

Methanesulfonate offers a number of hypothetical possibilities for use as a substrate for microbial growth and metabolism (Kelly and Baker 1990; Cook et al. 1999). It could provide sulfur for biosynthesis to any organism able to cleave the C-S bond under aerobic or anaerobic conditions; it could undergo oxidation to carbon dioxide and sulfate, yielding metabolic energy for growth from the oxidation of its methyl-groups, and even of the sulfonate moiety to sulfate; its methyl groups could provide carbon intermediates (e.g. formaldehyde) for methylotrophic bacteria; it might be used anaerobically either as a terminal electron acceptor for anaerobic respiration (e.g. by sulfate-reducing bacteria), as a substrate for fermentative disproportionation, or even as a substrate for methanogenesis from its methyl groups by archaeal methanogens.

Methanesulfonate as a sulfur source

Numerous longer-chain sulfonates are used as sulfur sources for growth by diverse bacteria, fungi and algae (Biedlingmeier and Schmidt 1983; Uria-Nickelsen et al. 1993a,b; Cook et al. 1999), but the use of methanesulfonate seems to be much more restricted. *Chlorella* used C_1–C_8-alkanesulfonates as sulfur sources but the variation in C-S bond stability of the different sulfonates appeared to limit the growth rate, so that methanesulfonate, the most stable sulfonate, was the poorest sulfur source (Biedlingmeier and Schmidt 1983). A number of aerobic bacteria (e.g. *Klebsiella* sp., *Comamonas acidovorans*, *Rhodococcus* sp., *Bacillus subtilis*, *Enterobacter aerogenes*, *Serratia marcescens*, *Escherichia coli* and fluorescent pseudomonads) can use methanesulfonate as a sulfur source (Seitz et al. 1993; Uria-Nickelsen et al. 1993a,b; Chih-Ching et al. 1995; Van der Ploeg et al. 1998; Cook et al. 1999; Reichenbecher and Murrell 1999), but we have found no reports of its use as a sulfur source under anaerobic conditions, even by bacteria able to use it aerobically (Cook et al. 1999). Recently *Pseudomonas aeruginosa* has been shown to desulfonate alkanesulfonates using a reduced flavin mononucleotide-dependent monooxygenase, which showed highest activity with methanesulfonate (Kertesz et al. 1999). This 381-amino acid protein was encoded by an 1145 bp open reading frame (ORF), the *msuD* gene, one of three comprising a desulfonation operon, *msuEDC*. Similarly, *Bacillus subtilis* has an operon which includes an ORF encoding for an aliphatic-sulfonate monooxygenase with a broad substrate range (Van der Ploeg et al. 1998).

Methanesulfonate as a respiratory electron acceptor, fermentation substrate, or methanogenic substrate

To date, no bacteria have been described that can degrade methanesulfonate in the absence of oxygen (Chih-Ching

et al. 1995; Lie et al. 1996, 1998; Cook et al. 1999). We, and others, have not yet succeeded in isolating denitrifying methylotrophs able to use methanesulfonate as carbon and sulfur source under anoxic conditions. The ability of some fermentative heterotrophs to degrade sulfonates other than methanesulfonate anaerobically as sources of biosynthetic sulfur must involve a hydrolytic split of the C-S bond that does not require oxygen, so the possibility of finding anaerobic methanesulfonate degraders cannot yet be excluded: further anaerobic studies are needed both to seek denitrifying methanesulfonate-methylotrophs and bacteria using methanesulfonate as a sulfur source under anoxic conditions. Hydrolysis of methanesulfonate to produce methanol has not yet been shown in any organism, but methanesulfonate hydrolysis as a means of splitting the C-S bond is possible:

$$CH_3SO_3^- + H_2O = CH_3OH + HSO_3^- \ (\Delta G^{\circ\prime} = -56.4 \text{ kJ mol}^{-1})$$

The standard free energy change for this reaction was estimated using a ΔG_f° value for the methanesulfonate ion ($\Delta G_f^\circ = -409.6$ kJ mol^{-1}), calculated by the group substitution method (Mavrovouniotis 1991), and published ΔG_f° values for the other compounds (Thauer et al. 1977; Mavrovouniotis 1990; Kelly 1999). The moderately exergonic value for the overall hydrolysis suggests that enzymatic hydrolysis of methanesulfonate is at least hypothetically feasible.

Methanesulfonate is an analogue of methanephosphonate, which has been shown to be degraded by *Comamonas* (formerly *Pseudomonas*) *testosteroni* to produce methane and phosphate by hydrolytic cleavage of the C-P bond (Daughton et al. 1979). The analogous reaction for methanesulfonate would be:

$$CH_3SO_3^- + H_2O = CH_4 + HSO_4^- \ (\Delta G^{\circ\prime} = -143.5 \text{ kJ mol}^{-1})$$

This has not yet been demonstrated, but the possible occurrence of the reaction, which is thermodynamically exergonic and hence does not present an obvious energetic problem, is worth investigating. Methanesulfonate has not yet been shown to be a respiratory electron acceptor for any sulfate-reducing bacterium, although some *Desulfovibrio* strains can use aminomethanesulfonate (Cook et al. 1999). Use of methanesulfonate or its hydrolysis products as respiratory hydrogen acceptors by the action of methanogens or sulfate reducers could conceivably lead to its anaerobic mineralization to methane and sulfite or sulfide:

$$CH_3SO_3^- + H_2 = CH_4 + HSO_3^- \ (\Delta G^{\circ\prime} = -152.5 \text{ kJ mol}^{-1})$$

$$CH_3SO_3^- + 4H_2 = CH_4 + HS^- + 3H_2O \ (\Delta G^{\circ\prime} = -348.4 \text{ kJ mol}^{-1})$$

Methylotrophic growth on methanesulfonate as a source of carbon and energy

Unlike all the higher sulfonates, whose breakdown leads to compounds containing at least C_2 units which can be degraded by central metabolic pathways (e.g. the Krebs' cycle), desulfonation of methanesulfonate can lead initially only to methanol or formaldehyde. Thus only methylotrophic bacteria (or yeasts) are likely to be able to use methanesulfonate as the sole substrate for growth, as any non-methylotrophic heterotroph possessing a methanesulfonate monooxygenase enzyme (Van der Ploeg et al. 1998; Kertesz et al. 1999) would not be able to derive cell-carbon exclusively from methanesulfonate, although the oxidation of methanesulfonate to carbon dioxide and water could act as a supplementary energy source. The possibility of deriving energy chemolithotrophically from the oxidation of sulfite released from methanesulfonate to sulfate exists, but this would allow only very limited autotrophic growth (Kelly 1999). The first bacterium isolated on methanesulfonate as sole substrate (Baker et al. 1991) was characterized as belonging to a novel genus within the α-Proteobacteria, *Methylosulfonomonas*, examples of which were readily isolated from various soils (Holmes et al. 1997). Novel marine isolates were also obtained, comprising another phylogenetically distinct genus within the α-Proteobacteria, *Marinosulfonomonas* (Thomp-

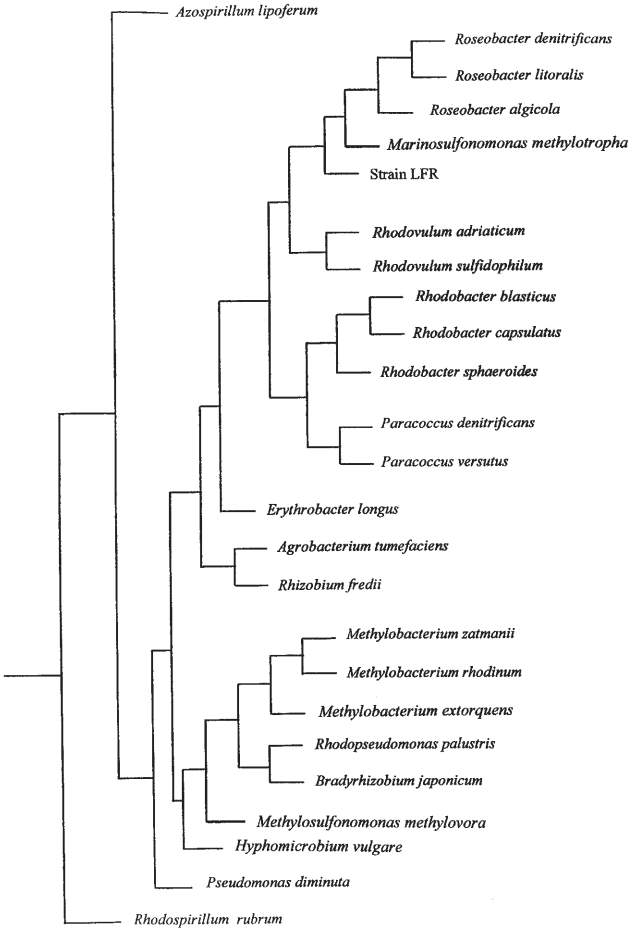

Fig. 1 Phylogenetic tree, based on 16S rRNA sequences, indicating the diversity of the four genera within the α-Proteobacteria which have been shown to contain strains able to use methanesulfonate as their growth substrate: *Marinosulfonomonas, Methylobacterium, Methylosulfonomonas* and *Hyphomicrobium*. (Based on Holmes et al. 1997)

son et al. 1995; Fig. 1). These observations, and the inability of some other well-known genera of methylotrophs to use methanesulfonate, suggested that methanesulfonate use might be restricted to these specialized genera. More recently, however, we have obtained some 20 new isolates that can grow on methanesulfonate: these have been characterized by 16 S rRNA sequence analysis, and are similar to the known species of *Hyphomicrobium* and *Methylobacterium* (Fig. 1; N. Baxter, P. De Marco, A.P. Wood and J.C. Murrell, unpublished data).

Mechanism of growth on methanesulfonate

All of the aerobic bacteria shown to date to use linear alkanesulfonates as growth substrates probably use a monooxygenase to split the C-S bond of the sulfonate, as was first observed with the degradation of C_4–C_8 alkanesulfonates by *Pseudomonas* strains (Thysse and Wanders 1974; Cook et al. 1999). This proved also to be the case for *Methylosulfonomonas* and *Marinosulfonomonas*, in which the initial cleavage of methanesulfonate was effected by an NADH-dependent monooxygenase (Kelly et al. 1994; Higgins et al. 1996):

$$CH_3SO_3^- + O_2 + NADH + H^+ \rightarrow HCHO + HSO_3^- + H_2O + NAD^+$$

Some of the formaldehyde is oxidized to carbon dioxide to provide NADH and energy for biosynthesis from the remaining formaldehyde, which is assimilated by the serine pathway (Fig. 2; Kelly et al. 1994; Thompson et al. 1995; Kelly and Murrell 1996). Sulfite is the other product of the oxygenase, and this is detectable chemically, but is converted to sulfate either by autooxidation or enzymatically by sulfite dehydrogenase. Hydrolysis of methanesulfonate to produce methanol does not occur in any of the methylotrophs isolated so far. Indeed, methanol is not involved as an intermediate in methanesulfonate breakdown, and mutants of *Methylosulfonomonas* lacking methanol dehydrogenase were found to be unimpaired in their ability to use methanesulfonate (Higgins et al. 1996). In addition, oxidation of methanesulfonate by *Marinosulfonomonas* strains was unaffected by cyclopropanol, which completely inhibited methanol oxidation (Thompson et al. 1995).

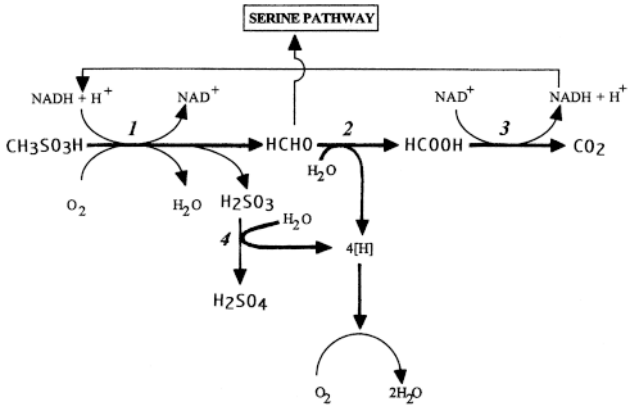

Fig. 2 Pathway for the oxidation of methanesulfonate and assimilation of intermediate formaldehyde via the serine pathway (Kelly et al. 1994; Thompson et al. 1995; Kelly and Murrell 1996; Murrell et al. 1996). Key reactions are: (1) NADH-dependent methanesulfonate monooxygenase; (2) formaldehyde dehydrogenase; (3) formate dehydrogenase, generating NADH which is consumed by the monooxygenase reaction; (4) the oxidation of sulfite to sulfate: this may be catalysed by sulfite dehydrogenase (as indicated in the scheme) or by autooxidation in solution. Conventional formulae are used to express methanesulfonic, formic, sulfurous and sulfuric acids, but under physiological conditions these would be present as their anions

The methanesulfonate monooxygenase of *Methylosulfonomonas* and *Marinosulfonomonas*

Our earlier work suggested the presence of a somewhat specific system for methanesulfonate metabolism in *Methylosulfonomonas methylovora* strain M2 and *Marinosulfonomonas methylotropha* strains TR3 and PSCH4, which when grown on methanesulfonate showed only limited ability to oxidize other sulfonates (Kelly et al. 1994; Thompson et al. 1995; Murrell et al. 1996). Strain M2 oxidized aminomethanesulfonate and ethanesulfonate at 86% and 36%, respectively, of the rate of methanesulfonate oxidation, but oxidized propanesulfonate at only 8% of that rate, and longer sulfonates not at all (Kelly et al. 1994). Similarly, strains TR3 and PSCH4 oxidized ethanesulfonate and propanesulfonate at 41–52% and 25–35%, respectively, of the methanesulfonate rates, but only TR3 oxidized butanesulfonate (at 24% of the methanesulfonate rate) and neither oxidized pentanesulfonate (Thompson et al. 1995).

Cell-free extracts of strain M2 contained a cytoplasmic methanesulfonate monooxygenase activity that was specifically induced by growth on methanesulfonate and catalysed methanesulfonate-dependent NADH (but not NADPH) oxidation with an NADH:methanesulfonate stoichiometry of 1 : 1 (Kelly et al. 1994; Higgins et al. 1996). This NADH oxidation was inhibited by metal chelators and by azide, cyanide and CO, indicating the involvement of metal ions and electron transport in the activity of the monooxygenase (Higgins et al. 1996; Kelly and Murrell 1996; Murrell et al. 1996), as is typical of other oxygenases (Mason and Cammack 1992). Crude extracts also showed substrate-dependent NADH oxidation with aminomethanesulfonate (36% of methanesulfonate-dependent rate), ethanesulfonate (79%) and propanesulfonate (29%) but not longer chain alkanesulfonates (Higgins et al. 1996).

By means of FPLC using Q-Sepharose, the methanesulfonate monooxygenase of strain M2 was resolved into three distinct fractions, none with individual methanesulfonate oxidizing activity, but which together were reconstituted into an active form (Higgins et al. 1996; Kelly and Murrell 1996). Activity of the reconstituted system was greatly stimulated by FAD and ferrous ions, suggesting these to be necessary for normal enzyme function, in

common with other oxygenases (Higgins et al. 1996). While these results indicate a specific iron site to be required for the monooxygenation, the molecular identity of the site (e.g. possibly as a mononuclear iron site like that of aromatic dioxygenases; De Marco et al. 1999) has not been proved. The substrate specificity of the reconstituted system was also much more restricted than that of the crude extract: ethanesulfonate and propanesulfonate supported NADH oxidation at 98% and 36%, respectively, of the methanesulfonate rate, but no other sulfonates, including aminomethanesulfonate, were used as substrates (Higgins et al. 1996; Kelly and Murrell 1996). The three fractions were subsequently further purified into four components, all of which were required for an active methanesulfonate monooxygenase (Murrell et al. 1996): (1) the putative hydroxylase of methanesulfonate monooxygenase: a 200 kDa complex of two major polypeptides of around 50 and 20 kDa; (2) a 16 kDa ferredoxin component; and (3) the putative reductase component of methanesulfonate monooxygenase: a 36–38 kDa monomeric protein catalysing the NADH-dependent reduction of several electron acceptors, including cytochrome *c* (Murrell et al. 1996; Higgins et al. 1997; De Marco et al. 1999).

Characterization of methanesulfonate monooxygenase

Recently, considerable progress has been made in the molecular characterization and genetics of the methanesulfonate monooxygenase complexes in *Methylosulfonomonas* and *Marinosulfonomonas* as well as the monooxygenases acting on methanesulfonate in *Pseudomonas aeruginosa* and *Bacillus subtilis* (Van der Ploeg et al. 1998; De Marco et al. 1999; Kertesz et al. 1999; Reichenbecher and Murrell, unpublished data). These are summarized in the following sections.

The hydroxylase component of the methanesulfonate monooxygenase of *Methylosulfonomonas methylovora*

This has been purified to homogeneity by FPLC using Q-Sepharose, hydroxyapatite, and Mono-Q columns (Reichenbecher and Murrell, unpublished results), and shown to consist of large (α) and small (β) subunits of molecular masses of 48 kDa and 20 kDa, respectively. These molecular masses were also confirmed by MALDI (Matrix-assisted laser desorption/ionisation) and FT-ICR (Fourier transform ion cyclotron resonance) mass spectrometry. The molecular masses for the α- and β-subunit polypeptides were also calculated as 48,473 Da and 20,478 Da, respectively, for the amino acid composition predicted by the DNA sequences of their respective coding genes (De Marco et al. 1999). The respective pI values for these α and β subunit sequences were 6.73 and 5.58 (De Marco et al. 1999). The native molecular mass of the protein, estimated by gel filtration, was about 200 kDa, indicative of an $\alpha_3\beta_3$ structure (molecular mass 206,853 Da, as calculated from the coding sequences) for the enzyme. Spectral analysis showed the presence of a Rieske [2Fe-2S] centre. For comparison, it is noteworthy that the naphthalene dioxygenase described by Kauppi et al. (1998) was an $\alpha_3\beta_3$ protein with Rieske and iron centres.

The ferredoxin component of the methanesulfonate monooxygenase of *Methylosulfonomonas methylovora*

The electron transfer protein was purified (74-fold) to homogeneity by Q-Sepharose ion exchange, S75 gel filtration, and Mono-Q ion exchange FPLC (Higgins et al. 1997). The purified protein had a native molecular mass of about 32 kDa, a pI of 3.9, and chemical and spectral analysis showed it to contain a Rieske [2Fe-2S] centre. Denaturing SDS-PAGE indicated the holoenzyme to comprise two identical subunits, and electron spray mass spectrometry showed a molecular mass of 13,752 Da (Higgins et al. 1997). This molecular mass was confirmed by that calculated for the predicted amino acid sequence (from the DNA sequence of the gene) as 13,748 Da (De Marco et al. 1999).

The reductase component of the methanesulfonate monooxygenase of *Methylosulfonomonas methylovora* strain M2

The reductase component has to date been partially purified by Q-Sepharose FPLC, but proved (like the reductases of other bacterial oxygenases) to be unstable during purification. It has been identified as a single polypeptide of molecular mass around 38 kDa, with a pI of about 6.5 (Reichenbecher and Murrell, unpublished data). Its molecular mass calculated from the DNA gene sequence was 38,852 Da (De Marco et al. 1999).

Molecular biology of methanesulfonate monooxygenase from *Methylosulfonomonas* and *Marinosulfonomonas*

N-terminal sequences for all four components of *Methylosulfonomonas* methanesulfonate monooxygenase were used to design oligonucleotide probes, enabling detection and cloning of the coding genes (Higgins et al. 1997; De Marco et al. 1999). The genes, designated *msmABCD*, were clustered on the *Methylosulfonomonas* chromosome (Fig. 3), probably constituting an operon for the coordinated expression of methanesulfonate monooxygenase, and transcribed into a single mRNA (De Marco et al. 1999). They were cloned on a 7.5 kb DNA fragment and total sequencing of this fragment enabled determination of the full sequence for each methanesulfonate monooxygenase component, and the calculation of molecular masses and pI values cited. The identity of the genes was confirmed by the match of the N-terminal sequences of

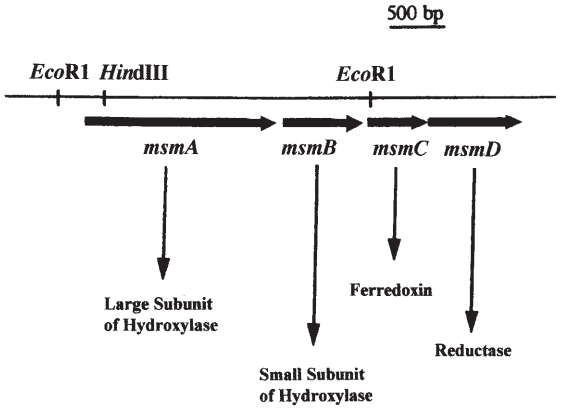

Fig. 3 Organization of the methanesulfonate monooxygenase gene cluster from *Methylosulfonomonas methylovora* (based on De Marco et al. 1999). The genes, *msmABCD*, and their products, the four components of methanesulfonate monooxygenase, are shown

the purified polypeptides with those indicated by the DNA sequences.

The genes for the large (α) and small (β) subunits of the hydroxylase component are designated *msmA* and *msmB*. *msmA* codes for a 414-amino acid polypeptide showing significant identity with the α subunits of hydroxylases known from other mono- and dioxygenases. It does, however, show novel features, including an unusually long 26-residue sequence between the two conserved histidine-cysteine residues of the Rieske motif (De Marco et al. 1999). This sequence in other oxygenases is usually no more than 18 residues. Other regions showing differences are those likely to be involved in recognition of the substrate methanesulfonate and in ligating a mononuclear iron centre for oxygen processing (De Marco et al. 1999). *msmB* codes for a 181-amino acid polypeptide and shows 44–51% similarity in the N-terminal region to the β subunits of several dioxygenases, but no similarity of the C-terminal region to other known oxygenase subunits (De Marco et al. 1999). No significant sequence identity has been found with the methane monooxygenase (MMO) of methane-oxidizing bacteria, and methanesulfonate is not a substrate for MMO. Detailed comment on methanesulfonate monooxygenase sequence identities and structure compared with other oxygenases, including several dioxygenases, was made by De Marco et al. (1999).

The gene for the ferredoxin component is *msmC*, which codes for a 122-amino acid polypeptide whose sequence shows a canonical motif characteristic of Rieske-type [2Fe-2S] centre-binding proteins (CXH-X_{17}-CXXH), and similar to those of some other bacterial ferredoxins (Higgins et al. 1997; De Marco et al. 1999). Phylogenetic comparisons showed the MsmC polypeptide to be most closely related to the toluene monooxygenases of *Pseudomonas* species (Higgins et al. 1997).

msmD codes for a 366-amino acid polypeptide, which is the reductase component of methanesulfonate monooxygenase, has a chloroplast-like [2Fe-2S] centre, and shows significant identity (FAD- and NAD-binding motifs) to the reductase components of known oxygenases (De Marco et al. 1999; Reichenbecher and Murrell, unpublished data). Purification of this protein to homogeneity, and the expression of *msmD* in a suitable expression host, have not yet been achieved.

Molecular analysis of *Marinosulfonomonas* revealed two copies of the methanesulfonate monooxygenase genes, which have been cloned and sequenced (Baxter N, Scanlan J and Murrell JC, unpublished data). Comparing the derived amino acid sequences with those for the corresponding genes from *Methylosulfonomonas* gave sequence similarities for *msmA*, *msmB*, *msmC* and *msmD* from each organism of 89%, 82%, 78% and 78%, respectively (Baxter et al. unpublished data).

Molecular genetics of methanesulfonate-oxygenases from *Pseudomonas aeruginosa* and *Bacillus subtilis*

Recently, Kertesz et al. (1999) showed an operon of three genes, *msuEDC*, in *Pseudomonas aeruginosa*, coding for the "sulfate starvation-induced proteins" (Van der Ploeg et al. 1998; Kertesz et al. 1999). This operon was part of the *cys* regulon, and the genes coded for proteins synthesized when the bacteria used organosulfur compounds such as methanesulfonate as sole sulfur sources. The *msuE* gene coded for a 186-amino acid protein, which was an NADH-dependent FMN reductase. The *msuD* coded for a 381-amino acid protein (41.6 kDa) that was related to several flavoprotein-type monooxygenases, including the SsuD proteins for sulfonate sulfur utilization from *E. coli* and *Bacillus subtilis* (Van der Ploeg et al. 1998; Kertesz et al. 1999) as well as showing 22–30% identity to dibenzothiophenesulfone monooxygenase and nitrilotriacetate monooxygenase (Kertesz et al. 1999). The MsuD protein showed greatest activity as a putative methanesulfonate monooxygenase and is one of several enzymes allowing use of methanesulfonate as a sulfur source by *P. aeruginosa*.

An *ssuD* gene also occurs in *Bacillus subtilis*, in which it is one of five genes organized in an operon-like structure (Van der Ploeg et al. 1998). The SsuD protein has 378 amino acids (41.4 kDa), essentially identical to that in *P. aeruginosa*, which showed highest sequence similarity (77%) to the SsuD protein (formerly Ssi6) from *E. coli* (Van der Ploeg et al. 1998). Both are apparently reduced FMN-dependent, aliphatic sulfonate monooxygenases, enabling desulfonation of sulfonates as sulfur sources for growth.

Molecular ecology of methanesulfonate-using bacteria and their detection using functional and phylogenetic gene probes

It has been possible to design gene-specific primers to analyse newly isolated methanesulfonate users from a variety of different environments by using the sequences determined for the highly conserved *msmA* from *Methylo-*

sulfonomonas strain M2 and *Marinosulfonomonas* strain TR3. This has resulted in tests on about 15 different methanesulfonate-using strains, identified as *Methylosulfonomonas*, *Marinosulfonomonas*, *Methylobacterium* and *Hyphomicrobium* by 16S rRNA sequence analysis, giving positive PCR products with the *msmA*-specific primer. Their *msmA* genes have subsequently been cloned and sequenced and found all to be 80–90% identical (at derived amino acid sequence level) to the MsmA polypeptide from *Methylosulfonomonas* strain M2. Remarkably, the 26 amino acid sequence centred around the Rieske centre is highly conserved and is therefore an excellent signature sequence motif for methanesulfonate monooxygenase. We have also been able to detect *msmA* in a variety of enrichment cultures using this PCR assay (Baxter et al. unpublished data). DNA was extracted directly from aerobic enrichment cultures, with methanesulfonate as the sole substrate, using inocula from soil, sediment and marine samples. These DNA samples were used as the DNA template in PCR assays using the *msm*A primer set. PCR products of the predicted size were obtained with DNA from all the enrichments tested. These were cloned into the In Vitrogen TA cloning vector to obtain libraries, each containing approximately 100 clones, of *msm*A sequences from soil, sediment and seawater enrichments. The majority of the sequences retrieved by PCR from the different enrichment cultures were very similar (up to 95% identity) to the *msmA* sequences of the methanesulfonate-utilizer type strains of *Methylosulfonomonas* and *Marinosulfonomonas* (Baxter et al. unpublished data). These observations strongly support the view that a conserved methanesulfonate monooxygenase enzyme is present in a variety of bacterial genera and consequently enables the use of molecular techniques for the direct study of environmental samples, thereby avoiding laborious and potentially biased enrichment culture methods (De Marco et al. 1999).

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