Comparison of pathways for biodegradation of monomethyl sulphate in *Agrobacterium* and *Hyphomicrobium* species

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Different mechanisms have been proposed previously for the biodegradation of monomethyl sulphate (MMS) in *Agrobacterium* sp. and *Hyphomicrobium* sp. Sulphate liberation from MMS in *Agrobacterium* sp. M3C was previously shown to be O₂-dependent, whereas in several *Hyphomicrobium* spp. the initiating step has been considered hitherto to be hydrolytic and catalysed by methyl sulphatase. In the present study, *Agrobacterium* and *Hyphomicrobium* strains were compared for their ability to oxidize MMS and its potential metabolites in the oxygen electrode. MMS-grown *Agrobacterium* sp. M3C and *Hyphomicrobium* sp. MS223 oxidized MMS with consumption of 0.5 mol O₂ per mol of substrate, but they were unable to oxidize methanol. By repeatedly challenging MMS-grown *Hyphomicrobium* with MMS in the electrode chamber, all the O₂ in the electrode became exhausted, at which point SO₄²⁻ liberation stopped although excess MMS was available. SO₄²⁻ release resumed immediately when O₂ was re-admitted to the electrode chamber. Thus liberation of SO₄²⁻ from MMS in the oxygen electrode was dependent on the continuing availability of O₂. *Hyphomicrobium* sp. MS223 therefore closely resembled *Agrobacterium* sp. M3C in its obligatory requirement for O₂ in MMS degradation. Unlike *Agrobacterium* sp. M3C, *Hyphomicrobium* sp. MS223 was able to grow on methanol and methanol-grown cells oxidized methanol (0.5 mol O₂ per mol of substrate) but not MMS. Cyclopropanol, an inhibitor of methanol dehydrogenase, abolished oxidation of methanol by methanol-grown *Hyphomicrobium* sp. MS223 but did not affect oxidation of MMS by MMS-grown cells. Thus *Hyphomicrobium* sp. MS223 expresses enzymes for oxidation of methanol when needed for growth on this compound, but not when grown on MMS. These results are consistent with the absence of methanol from the pathway for biodegradation of MMS by *Hyphomicrobium* sp. MS223 and suggest that in at least some *Hyphomicrobium* spp. an oxidative mechanism initiates biodegradation.

Introduction

Monomethyl sulphate (MMS) enters the environment as a component in fly-ash from coal- and oil-burning power stations (Eatough *et al.*, 1981; Lee *et al.*, 1980) and as a waste byproduct of the use of dimethyl sulphate in industrial methylation processes (Ghisalba *et al.*, 1985a). Dialkyl sulphates are very reactive and transfer the first alkyl group readily to acceptors such as OH and NH functional groups, but the residual monoalkyl sulphates are (relatively) much more stable (Heiner *et al.*, 1962). Studies of the bacterial biodegradation of MMS are thus pertinent to assessing its environmental impact and acceptability, and to the development of methods for industrial waste-treatment which might replace current methods such as incineration (Ghisalba *et al.*, 1985a).

Mechanisms of bacterial biodegradation of MMS are also of particular fundamental interest because this compound lies at the interface between two groups of organic compounds which separately have attracted considerable attention and for which metabolic pathways are quite distinct. On the one hand, MMS is a C₁ compound and thus a potential addition to the list of compounds that support growth of methylotrophic organisms (Anthony, 1982; Higgins *et al.*, 1980; Large, 1983). On the other hand it is a sulphate ester, in fact the shortest in the homologous series of linear primary alkyl sulphates, the longer of which are surfactants. Much is now known about the biochemical mechanisms and enzymology of alkyl sulphate biodegradation. Long-chain esters undergo hydrolysis catalysed by alkyl sulphatases in *Pseudomonas* sp. (Bartholomew *et al.*, 1978; Bateman *et al.*, 1986; Cloves *et al.*, 1980; Dodgson & White, 1983; Dodgson *et al.*, 1982; Matcham *et al.*, 1977a, b; Shaw *et al.*, 1980; White & Russell, 1993) but these enzymes cease to function when the alkyl chain

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Abbreviation: MMS, monomethyl sulphate.

length falls below C_5 . A coryneform species has been shown recently to produce an alkyl sulphatase restricted to primary esters in the C_3 – C_7 chain length range (White & Matts, 1992). The degradation of the shortest secondary alkyl sulphate, propyl 2-sulphate, is initiated by oxidation of a methyl group to lactate 2-sulphate before a specific D-lactate-2-sulphatase removes inorganic sulphate (Crescenzi *et al.*, 1984, 1985).

A methyl sulphatase has been reported to initiate biodegradation of MMS in *Hyphomicrobium* spp. (Ghisalba *et al.*, 1985a, 1986; Ghisalba & Kuenzi, 1983; Schar & Ghisalba, 1985), based on the detection of methanol in culture fluids during growth on the ester and on the ability of *Hyphomicrobium* spp. to produce methanol dehydrogenase (Schar *et al.*, 1985). In contrast, the pathway proposed for MMS biodegradation in *Agrobacterium* sp. M3C is initiated by monooxygenase-catalysed conversion of the ester to formaldehyde without formation of methanol. This mechanism was based on results of oxygen electrode studies and on the dependence of SO_4^{2-} release on the availability of O_2 . The present study was undertaken to make a closer comparison of the pathways for MMS biodegradation in *Hyphomicrobium* and *Agrobacterium* species.

Methods

Chemicals. Except where otherwise stated, all chemicals were of analytical grade supplied by Merck (BDH) or Sigma. Noble agar was obtained from Difco, and helium from British Oxygen Corporation. MMS was prepared from dimethyl sulphate (Fluka) as described elsewhere (Davies *et al.*, 1990). Elemental composition determined by combustion analysis was carbon, 8.06% (theoretical 7.99%), and hydrogen, 1.99 (2.01%), indicating a purity of 99%. The infra-red spectrum contained strong absorption bands at 1210 cm^{-1} and $770\text{--}810\text{ cm}^{-1}$ corresponding to vibrational modes in S–O and C–O–S bonds of $R\text{--}O\text{--}SO_3^-$ (Lloyd *et al.*, 1961). Spectra lacked absorption bands at 3320 cm^{-1} (unsubstituted hydroxyl groups) as would be found in methanol. The natural-abundance ^{13}C -NMR spectrum (100.6 MHz, Bruker Aspect 2000) of MMS gave a single resonance (58.2 p.p.m.) with no sign of resonance at 50 p.p.m. characteristic of methanol. HPLC ion chromatography of the sample (see below) yielded a single major peak, and contamination of the sample by SO_4^{2-} so low as to be indistinguishable from that in the Type I water used to prepare the sample.

Cyclopropanol, as a 4 mM solution in water, was kindly supplied by Professor I. Ochura, Department of Bioengineering, Tokyo Institute of Technology, Japan.

Where samples of incubation mixtures were to be analysed by ion chromatography, the water used for the preparation of all solutions was Type 1 deionized 18 M Ω , 0.22 μm -filtered water from a Millipore Q50 water-purification unit.

Organisms and culture conditions. *Agrobacterium* sp. strain M3C (NCIMB 13265) was isolated previously in Cardiff for its ability to grow on MMS (Davies *et al.*, 1990) as sole source of carbon. *Hyphomicrobium* sp. strain MS223 (NRRL-B-12570, Agricultural Research Culture Collection, Preoria, Illinois 61604, USA) was originally isolated in Basel, also for its ability to utilize MMS as sole source of carbon (Ghisalba & Kuenzi, 1983). A culture of this isolate was kindly supplied by Dr O. Ghisalba, CIBA Geigy Corporation,

Basel, Switzerland. Axenic culture was established by repeated subculturing on minimal-medium agar plates containing MMS as sole source of carbon.

For the present study culture media for both *Agrobacterium* and *Hyphomicrobium* spp. contained, per litre of distilled water: NH_4NO_3 , 2.0 g; Na_2HPO_4 , 1.4 g; KH_2PO_4 , 0.6 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g; and 1 ml of trace elements solution (Ghisalba & Kuenzi, 1983). Trace elements solution contained 20 mg each of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per litre of distilled water. This basal salts medium (45 ml in 100 ml Erlenmeyer flasks) was autoclaved at 0.1 MPa, 121 °C for 20 min. MMS or methanol (0.33 M) dissolved in 1 M-phosphate buffer pH 7, was filter-sterilized and added aseptically to the basal salts medium to give a final concentration of 33 mM. The additional phosphate buffer (0.1 M final concentration) was necessary to accommodate significant release of H^+ during utilization of MMS (Ghisalba *et al.*, 1985a; Ghisalba & Kuenzi, 1983) and other short chain sulphate esters (Crescenzi *et al.*, 1985). Cultures were incubated at 30 °C in an orbital shaker (150 r.p.m.) and growth was monitored by culture optical density (OD_{420} , LKB-Pharmacia Novaspek II spectrophotometer). Stock cultures were maintained on slopes of the same medium solidified with 1.5% (w/v) Noble agar.

Preparation of cell suspensions. Cells at the end of the exponential phase of growth (OD_{420} in the range of 0.4–0.7) were harvested by centrifugation (90 000 g_{av} , min, GS3 rotor in a Sorval RC-5B refrigerated centrifuge), washed at least four times and resuspended in phosphate buffer pH7, usually to one-tenth of the original culture volume.

Analysis of MMS and SO_4^{2-} . MMS and SO_4^{2-} were analysed by ion chromatography with suppressed conductivity detection. Samples were individually injected onto an Omnipac PAX-500 column installed in a DX300 series ion chromatography system (Dionex). The column was eluted isocratically at 1 ml min^{-1} with eluent containing 30 mM-sodium hydroxide (low in carbonate) and 5% (v/v) methanol (HiPerSolv grade) which was initially degassed and stored under helium, to prevent the elevation of carbonate ions from dissolved CO_2 and the production of gas bubbles in the HPLC detector. Peak areas were integrated using a Spectra Physics 4400 integrator and were corrected for any temperature drift during the elution. The correction factor for this particular equipment was 1.7% per °C, and all values were adjusted to correspond to 25 °C. Integrated temperature-corrected peak areas were then converted to molarity of MMS and SO_4^{2-} using separately determined calibration curves.

O_2 -uptake measurements. *Agrobacterium* sp. strain M3C grown on MMS or *Hyphomicrobium* sp. strain MS223 grown on MMS or on methanol were harvested at the end of the exponential phase, washed in 0.1 M-phosphate buffer pH 7, and resuspended at 10-fold concentration as described above. Samples of the concentrated cell suspension (0.5 ml) were introduced into a Clark-type oxygen electrode (Rank Brothers) containing 2.7 ml 0.1 M-phosphate buffer equilibrated with air at 30 °C. The rate of O_2 -uptake of the cells was recorded for 5 min to establish a basal rate, before addition of concentrated solutions of MMS or other test compounds (typically 20 μl , 200 nmol). The electrode was calibrated with NADH as described by Robinson & Cooper (1970).

O_2 -uptake and liberation of SO_4^{2-} . Water used for the preparation of all solutions in these experiments was Type 1 deionized 18 M Ω , 0.22 μm -filtered water. *Agrobacterium* sp. strain M3C and *Hyphomicrobium* sp. strain MS223 were harvested at the end of the exponential phase and washed in 5 mM-phosphate buffer pH 7. Cells were resuspended in one-tenth of the original culture volume, and 1 ml aliquots of the resuspensions were added to 2.7 ml of aerated 5 mM-phosphate buffer in the oxygen electrode. The rate of O_2 -uptake was recorded for a period prior to the addition of MMS to establish the

Fig. 1

Fig. 2

Fig. 1. O_2 -uptake by MMS-grown cells of *Agrobacterium* sp. M3C. Substrates (200 nmol aliquots) were added at the indicated points without allowing the entry of O_2 into the electrode chamber.

Fig. 2. O_2 -uptake by MMS-grown cells of *Hyphomicrobium* sp. MS223. Substrates (200 nmol aliquots) were added at the indicated points without allowing the entry of O_2 into the electrode chamber.

basal rate of O_2 -uptake. MMS (50 μ l, 670 nmol) was then added to the reaction chamber. At intervals both before and after addition of MMS, aliquots (50 μ l) were withdrawn from the electrode chamber without the admission of atmospheric O_2 by using a microsyringe. Each aliquot was immediately diluted (40 μ l to 1 ml) with water, filtered through a 0.22 μ m Anotop filter (Whatman) and frozen at -20°C to await subsequent analysis for MMS and SO_4^{2-} contents.

When the O_2 -uptake rate had returned to the basal rate, a further aliquot of MMS was added to the chamber and samples were taken as before. Previous experiments had shown that the amount of MMS which had been added in these two additions was enough to make the reaction chamber anaerobic. With the reaction chamber in this state, two further aliquots of MMS (50 μ l, 670 nmol) were added, and samples taken as before. The stopper of the oxygen electrode was then removed to allow atmospheric O_2 to dissolve into the solution. When the O_2 concentration reached about 80% saturation the stopper was replaced and a further four samples were taken for analysis.

Results

Both *Agrobacterium* sp. strain M3C and *Hyphomicrobium* sp. strain MS223 grew substantially (maximum $OD_{420} = 1.2$) and at about the same rate (doubling time approximately 8 h) on 33 mM-MMS. However, only *Hyphomicrobium* sp. strain MS223 was capable of growth

on methanol (33 mM), with a doubling time of about 6 h and final OD_{420} of 1.5.

Agrobacterium sp. strain M3C grown on MMS (Fig. 1) and *Hyphomicrobium* sp. strain MS223 also grown on MMS (Fig. 2) each utilized about 1 mol O_2 for every 2 mol MMS added to the oxygen electrode chamber. When potential C_1 intermediates of the metabolic pathway were added to the electrode chamber (viz. methanol, formaldehyde and formate), the rate of oxygen uptake did not change from the basal rate for either isolate. In both organisms, the MMS-stimulated O_2 -uptake was not affected by the presence of cyclopropanol (0.4 mM final concentration), a known inhibitor of PQQ (pyrroloquinoline quinone)-dependent methanol dehydrogenase (Dijkstra *et al.*, 1984; Frank *et al.*, 1989; Shimoda & Okura, 1991).

O_2 -uptake by methanol-grown *Hyphomicrobium* sp. strain MS223 showed no deflection from the basal rate when MMS was added to the oxygen electrode chamber (Fig. 3). However, when the growth substrate, methanol, was added to the reaction chamber there was a sharp acceleration of O_2 -uptake. The additional utilization of O_2 was equivalent to about 1 mol O_2 utilized for every

Fig. 3. O_2 -uptake by methanol-grown cells of *Hyphomicrobium* sp. MS223. Substrates (200 nmol aliquots) were added at the indicated points without allowing the entry of O_2 into the electrode chamber. Cyclopropanol was added to give a final concentration of 0.4 mM.

2 mol methanol added to the chamber. The methanol-stimulated O_2 -uptake was abolished when cyclopropanol (0.4 mM final concentration) was added (Fig. 3).

Fig. 4 shows O_2 -uptake when serial additions of MMS were made to MMS-grown *Hyphomicrobium* sp. strain MS223. Simultaneously, the oxygen electrode chamber was sampled and analysed for residual MMS and SO_4^{2-} production. After the first addition, MMS was converted almost stoichiometrically to SO_4^{2-} , with the expected consumption of 0.5 mol O_2 per mol of ester added. After a second addition (29 min), the oxygen electrode became depleted of O_2 (42 min) and after this point relatively little conversion of MMS to SO_4^{2-} occurred, despite continued presence of MMS from further additions at 51 and 75 min. Biotransformation of MMS to SO_4^{2-} resumed at an appreciable rate only when O_2 was re-admitted to the electrode chamber (96 min).

Discussion

Two possible metabolic pathways for the degradation of monomethyl sulphate have been formulated (Fig. 5). Davies *et al.* (1990) proposed that the initial step in the degradation of MMS involved a monooxygenase enzyme, which oxidized MMS to methanediol monosulphate via a 4-electron transfer. The proposed mechanism showed that the electrons were derived equally from NADH and MMS and, because oxidation of NADH is subsumed in the basal rate, the addition of MMS to cells was expected to increase demand for electrons by $2e^-$ per molecule added. The methanediol sulphate produced from the initial step decomposed spontaneously to form SO_4^{2-} and formaldehyde, a central intermediary metabolite in C_1 metabolism (Attwood &

Quayle, 1984). The formaldehyde can either be assimilated by the organism via the serine pathway or oxidized to CO_2 via formate in sequential 2-electron transfers.

The alternative proposed pathway (Ghisalba & Kuenzi, 1983) involved the hydrolytic cleavage of MMS by a sulphatase enzyme to yield methanol and SO_4^{2-} . The methanol was then oxidized to formaldehyde via a 2-electron step by a PQQ-dependent methanol dehydrogenase enzyme (Schar *et al.*, 1985). The formaldehyde produced by this pathway then undergoes the same fate as that from the monooxygenase pathway.

The present study has shown that *Agrobacterium* sp. strain M3C grown on MMS required 0.5 mol O_2 per mol MMS added to resting cells in the oxygen electrode, i.e. a 2-electron transfer for each molecule of MMS. Bearing in mind that these cells did not oxidize exogenously added formaldehyde or formate, the 2-electron transfer was consistent with the operation of either the monooxygenase pathway (2-electron transfer prior to sulphate release) or the hydrolytic pathway (2-electron transfer after sulphate release) up to the formaldehyde stage. Previous work (Davies *et al.*, 1990) has established that sulphate release from MMS in *Agrobacterium* sp. strain M3C is dependent upon the immediate availability of O_2 . This, together with the inability of *Agrobacterium* sp. strain M3C to grow on methanol and the inability of MMS-grown cells to oxidize methanol, argues in favour of the oxygenolytic pathway (Fig. 5) for degradation of MMS in this isolate.

Hyphomicrobium sp. strain MS223 resembles *Agrobacterium* sp. strain M3C in its capacity for growth on MMS and its ability to oxidize MMS in the oxygen electrode (2-electron transfer per molecule of MMS, Fig. 2). Moreover, the conversion of MMS to SO_4^{2-} only occurred when molecular O_2 was freely available (Fig. 4). As with *Agrobacterium* sp. strain M3C, *Hyphomicrobium* sp. strain MS223 grown on MMS was unable to oxidize methanol in the oxygen electrode. The similarity in results for the two strains argues for the oxygenolytic cleavage mechanism in both cases.

Hyphomicrobium sp. strain MS223 and *Agrobacterium* sp. strain M3C differed significantly in the ability of the former, but not the latter, to grow on methanol. Methanol-grown cells of *Hyphomicrobium* sp. strain MS223 were capable of oxidizing methanol in the oxygen electrode (0.5 mol O_2 per mol methanol). This 2-electron transfer corresponds to oxidation of methanol to formaldehyde but no further, and is consistent with failure of exogenous formaldehyde or formate to elicit a response from the same cells in the electrode. Methanol oxidation by methanol-grown cells was inhibited by cyclopropanol, a known inhibitor of PQQ-dependent methanol dehydrogenase (Dijkstra *et al.*, 1984; Frank *et al.*, 1989; Shimoda & Okura, 1991). These results are in agreement with observations of O. Ghisalba's group

Fig. 4. Simultaneous O_2 -uptake and desulphation of MMS by *Hyphomicrobium* sp. MS223. Additions of MMS (50 μ l, 670 nmol) were made at the times indicated by the vertical dotted lines (3, 29, 51 and 75 min). The electrode chamber was opened to readmit oxygen at the vertical dashed lined (96 min), and closed again at 102 min. —, O_2 concentration; ●, MMS; ○, SO_4^{2-} .

Fig. 5. Hydrolytic and oxidative mechanisms for bacterial biodegradation of MMS.

(Ghisalba *et al.*, 1985a, b; Ghisalba & Kuenzi, 1983; Schar *et al.*, 1985) for growth on, and oxidation of, methanol. Evidently *Hyphomicrobium* sp. strain MS223 can synthesize enzyme(s) for the oxidation of methanol when the situation demands it (e.g. growth on methanol as sole source of carbon). However, in our hands the capacity to oxidize methanol was not expressed during growth on MMS. Moreover, although methanol oxidation by methanol-grown cells was inhibited by cyclopropanol, MMS oxidation by MMS-grown cells was unaffected by cyclopropanol. This provides strong evidence for the absence of methanol from the catabolic

pathway for MMS, and thus lends further weight to the operation of the oxygenolytic pathway (Fig. 5) rather than the hydrolytic pathway proposed hitherto (Ghisalba *et al.*, 1985a, 1986; Ghisalba & Kuenzi, 1983; Schar & Ghisalba, 1985).

Our data for *Hyphomicrobium* sp. MS223 are thus inconsistent with a sulphatase-initiated metabolism of MMS. The claim for the existence of a hydrolytic pathway in a group of MMS-degrading *Hyphomicrobium* spp. has been repeated in several papers (Ghisalba *et al.*, 1985a, 1986; Ghisalba & Kuenzi, 1983; Schar & Ghisalba, 1985) but the data supporting the generalization are limited. Methanol was detected (Ghisalba *et al.*, 1985a) in stirred-tank reactors in which MMS was used to support growth of *Hyphomicrobium* sp. MS72 (NRRL-B-12573). It may be significant that, in several of its biochemical characteristics, this particular isolate was different from other MMS degraders, including *Hyphomicrobium* sp. MS223 used in the present study (Ghisalba *et al.*, 1985b; Ghisalba & Kuenzi, 1983). Thus, while the conclusion that methanol was formed via a methyl sulphatase may be valid for *Hyphomicrobium* sp. MS72, the extrapolation to the other strains of *Hyphomicrobium* spp. is difficult to sustain, at least on the basis of published data. *Hyphomicrobium* sp. MS223 does synthesize a methanol dehydrogenase (Schar *et al.*, 1985) but this was produced during growth on methanol. To the best of our knowledge there is no published data to indicate production of methanol dehydrogenase during growth on pure MMS.

Location of the MMS-degrading activity in the cell-membrane of *Hyphomicrobium* spp. has been noted (Ghisalba *et al.*, 1986), and this location has hindered attempts to purify and characterize the enzyme. In contrast, all alkyl sulphatases studied to date are soluble and located either in the cytosol or in the periplasmic space (Dodgson *et al.*, 1982; Thomas *et al.*, 1988). On the other hand, some monooxygenases are known to be

located in the cell membrane (Large, 1983; Fox *et al.*, 1990; Pilkington & Dalton, 1990). Thus in terms of cellular localization the MMS-degrading enzyme is closer to the monooxygenases than to sulphatases. Efforts are currently underway to establish and compare MMS-degrading activities in cell-free extracts of *Agrobacterium* and *Hyphomicrobium*.

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