SHORT COMMUNICATION

Methyl Chloride: Naturally Occurring Toxicant and C-1 Growth Substrate

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A strain of *Hyphomicrobium* that could utilize methyl chloride as the sole carbon and energy source for growth was isolated from industrial sewage. The methylotroph utilized methyl chloride quantitatively with stoichiometric release of chloride ion. A specific growth rate (μ) of 0.09 h^{-1} was observed with about 1% (v/v) methyl chloride in the gas phase.

INTRODUCTION

Methyl chloride is a naturally occurring compound (see Harper, 1985; Singh *et al.*, 1983; White, 1982), which has been classified by the US Environmental Protection Agency as a priority pollutant (Keith & Telliard, 1979). It is a very water-soluble gas at normal temperature and pressure (Weast, 1984) and is used industrially as a methylating agent for silicones and lead (Edwards *et al.*, 1982). Methyl chloride is known to be detoxified in the liver (Kornbrust & Bus, 1983), to be oxidized by a methanotroph (Stirling & Dalton, 1979) and by isolated methane monooxygenase (Patel *et al.*, 1982), and to be enzymically hydrolysed (Keuning *et al.*, 1985), but it does not serve as a carbon source for growth (Stirling & Dalton, 1979; Janssen *et al.*, 1985) and further information on its biological fate is lacking (Watson *et al.*, 1980).

We have now isolated a bacterium that utilizes methyl chloride as a sole and growth-limiting source of carbon and energy.

METHODS

Materials. The inoculum was sludge from two industrial sewage plants (Schweizerhalle, Switzerland, and Grenzach, Germany). Methyl chloride ($\geq 99.5\%$, v/v) was purchased from Matheson; it was chromatographically pure and its identity was confirmed by mass spectrometry. Methane ($> 99.9\%$, v/v) and ethyl chloride (99.5% , v/v) were from Matheson. Other chemicals were from Fluka and were of the highest purity available. Liquid cultures were grown in screw-cap Erlenmeyer flasks closed with Mininert valves (Precision Sampling), which allowed ready sampling of the gas and liquid phases.

Growth. Cultures were grown in 100 mM-potassium phosphate buffer, pH 7.2, containing 25 mM-(NH₄)₂SO₄, 0.25 mM-MgSO₄ and trace elements (Cook & Hütter, 1981) to which a carbon source was added. Gases could be sterilized by filtration (0.2 μm pore diameter membrane filter) but we were able to draw gas from cylinder outlets by syringe and avoid contamination on injection of gas into the flask through the septum. Cultures were grown at 30 °C on an orbital shaker and the volume of air above the culture was tenfold larger than the culture volume. Solid media were prepared by addition of 1% (w/v) agar (Oxoid no. 1). Methyl chloride-minimal medium plates were incubated in desiccators with substrate (1%, v/v) in the gas phase. The substrate spectrum was examined in closed flasks, because the organism obviously grew with traces of carbon sources in the air.

To obtain growth yields, cultures containing different concentrations of substrate were allowed to grow into the stationary phase. Protein was measured and substrate exhaustion was confirmed. The yield was the slope of the line 'protein concentration' vs 'initial substrate concentration', and the correlation coefficient r was ≥ 0.985 (7

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points). Cells for use in non-growing suspensions or in the preparation of cell extracts were harvested in the late exponential phase, washed and stored frozen; cells were disrupted in a French press (Stucki *et al.*, 1981).

Enrichment and isolation of organisms. The inoculum was used directly to inoculate a 30 ml culture in a closed 300 ml Erlenmeyer flask containing 90 μ mol methyl chloride with, in addition, 250 μ mol methane as a putative major carbon source. The culture grew, with disappearance of methyl chloride, within 7 d and was transferred to identical fresh medium. After three successful transfers, the properties of the system were examined and pure cultures were isolated (see Results and Discussion). The organism has been submitted to the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG) where an accession number is pending.

Analytical methods. Growth was quantified as protein (Cook & Hütter, 1981). Growth rates were calculated from measurements of OD₅₄₆. Dechlorination by whole cells and cell extracts were measured as release of Cl⁻, determined colorimetrically (Bergman & Sanik, 1957) and amperometrically (Hartmans *et al.*, 1985). Methyl chloride and other gases were determined by gas chromatography (Stucki *et al.*, 1981). Dissolved O₂ was measured polarimetrically (S3 Monitor; Yellow Springs Instruments).

RESULTS AND DISCUSSION

The enrichment culture grew in the presence of methyl chloride and there was total disappearance of methyl chloride as well as extensive release of Cl⁻. Since methyl chloride was chemically stable under the conditions of the enrichment culture, and non-methylotrophs (strains A and 99; Cook & Hütter, 1981) did not cause decay of methyl chloride, methyl chloride was not decomposing due to chemical methylation of bacteria. The culture did not grow with methane as the sole carbon source but growth in methyl chloride-minimal medium was observed. A pure culture, designated MC1, was obtained by alternately growing on methyl chloride-minimal agar and in selective liquid culture in methyl chloride-minimal medium. Purity (uniformity of colony morphology) was then confirmed on plates with methanol-minimal medium. Growth with methyl chloride as a source of carbon would appear to be unusual. Three organisms contain enzymes that dechlorinate the compound, but it does not serve as a growth substrate (Stirling & Dalton, 1979; Patel *et al.*, 1982; Janssen *et al.*, 1985; Keuning *et al.*, 1985), and a *Hyphomicrobium* sp. that utilizes methylene chloride does not use methyl chloride (Stucki *et al.*, 1981).

Strain MC1 had a very narrow substrate spectrum. Methyl chloride, methanol, formate and ethanol were good substrates for growth whereas traces of growth were observed with acetate, butyrate and 3-hydroxybutyrate. Methane, methylene chloride, methylamine, ethyl chloride, 1,2-dichloroethane, pyruvate, glycerol, propionate, succinate and citrate did not support growth and the organism did not grow anaerobically in methanol-minimal medium containing nitrate.

Phase-contrast and electron microscopy (Fig. 1) revealed that strain MC1 was pleomorphic and Gram-negative. Plump motile rods with single sub-polar flagella were observed. Non-motile monoprosthecate rods with buds on the ends of the prostheca were also observed. These characteristics, together with methylotrophy, enable assignment of the strain to the genus *Hyphomicrobium* (Harder & Attwood, 1978).

The pure strain of *Hyphomicrobium* quantitatively utilized 90 μ mol methyl chloride and released a mean of 88 ± 3 (SD) μ mol Cl⁻, which was identified by two independent methods. The yield of strain MC1 with methyl chloride, 5 g protein (mol C)⁻¹, lay between the yield with methanol [7 g protein (mol C)⁻¹; a normal value (Anthony, 1982)] and the 2 g protein (mol C)⁻¹ observed with formate. The lower value with methyl chloride than with methanol implies that the product of dechlorination is formaldehyde and not methanol.

Substrate utilization (measured as release of Cl⁻) was concomitant with growth (Fig. 2, inset). The specific growth rate, however, was dependent on the substrate concentration. A maximum growth rate of about 0.09 h⁻¹ was observed at about 3 mM-methyl chloride (1%, v/v; Fig. 2); with 10% (v/v) substrate in the gas phase the rate was 0.04 h⁻¹. Growth rates of 0.12 and 0.04 h⁻¹ were observed with methanol and formate, respectively.

The mechanism of dechlorination has not been elucidated. Washed cell suspensions of methyl chloride-grown cells, but not of methanol-grown cells, dechlorinated methyl chloride in an oxygen-dependent reaction, but there was no consumption of methane. We presume that oxidation of methyl chloride to formaldehyde by methane monooxygenase is not involved (cf.

Fig. 1. Transmission electron micrograph of *Hyphomicrobium* sp. strain MC1. The organism was grown in methyl chloride-minimal medium. Samples on carbon-coated Formvar films were air-dried and unidirectionally shadowed with 3 nm platinum/carbon at an angle of 45°. The bar represents 1 μ m.

Fig. 2. Specific growth rate of *Hyphomicrobium* sp. strain MC1 as a function of substrate (methyl chloride) concentration. Each culture (150 ml) was inoculated with cells that were growing exponentially in homologous medium. Growth rates were estimated by following the increase in OD_{546} for at least three doublings. Substrate concentrations were kept within 0.2% of the values shown. Inset is the release of Cl^{-} during growth with about 2% (v/v) methyl chloride added to the gas phase.

Stirling & Dalton, 1979). Hydrolytic dehalogenation (Keuning *et al.*, 1985) was not observed, and methanol, which was rapidly oxidized by methanol-grown cells, was only slowly oxidized by methyl chloride-grown cells. We suspect that methanol is not an intermediate in the degradation of methyl chloride and that there may be a methyl chloride monooxygenase analogous in reaction to methane monooxygenase.

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