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Methane production and consumption in a cultivated humisol

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Summary. Laboratory studies were conducted on a cultivated humisol containing populations of both methanotrophs and methanogens. The molar ratio CO_2 produced: O_2 consumed: CH_4 consumed was 0.27:1.0:1.0. Methane oxidation showed typical Michaelis-Menten kinetics with apparent K_m values for CH_4 and O_2 of 66.2 μ*M* and 37.0 μ*M*, respectively. The low CO_2 yields and the effects of low dissolved oxygen indicated the presence of aerobic obligate methanotrophs. It is suggested that the methanotrophs in this soil are not entirely dependent on atmospheric CH_4 for growth and survival in situ.

Key words: Methane metabolism – Methanotrophs – Kinetics of methane uptake – Humisol

Methylotrophic bacteria use reduced carbon compounds containing no carbon-carbon bonds as sole sources of energy and carbon (Anthony 1982). This group is further divided on the basis of their ability to use methane in addition to methanol, dimethylether, methylated formate and carbonate (obligate methanotrophs) or complex carbon compounds (facultative methanotrophs) for growth (Whittenbury et al. 1975a, b; Haber et al. 1983).

Methanotrophs are recognized as an important component of the carbon cycle by regulating biogenic emissions of methane to the atmosphere (Romanovskaya et al. 1977; Higgins et al. 1981). Most quantitative studies on methane oxidation in the environment, however, have been restricted to aquatic systems (Rudd and Taylor 1979; Hanson 1980). Few reports are available for soil systems (e.g. Adamse et al. 1972) although methane oxidation by soil bacteria was established as early as 1906 (Zobell 1946). Forest and agricultural soils are of particular interest since the presence of methane oxidizers could signify dependence on atmospheric sources of methane for growth. Some studies have shown, for example, that swamp soils under drought conditions and certain tropical and temperate forest soils are significant sinks of atmospheric methane (Harriss et al. 1982; Keller et al. 1983).

We report here laboratory studies of methane production and the activity and kinetics of methane oxidizers in a cultivated humisol (organic muck soil).

Materials and methods

Soil. The soil used in this study was a cultivated humisol collected at the experimental farm of Macdonald College of McGill University, Ste. Anne de Bellevue, Quebec. Air-dried surface (0–10 cm) soil was sieved through a 2-mm mesh and stored at 4 °C. The soil had a pH of 6.8, moisture content of 69.0% (w/w) and bulk density of 0.3 g cm^{-3}. The organic content, measured as weight loss on ignition (550 °C) of oven-dried samples, was 43.7% (w/w). The soil contained 12 μg NH_4^+–N g^{-1} and 79 μg NO_3^-–N g^{-1} when extracted with 2 *N* KCl. No detectable NO_2^- was present.

Assay conditions. Five-gram portions of air-dried (3.36 g oven-dried) soil were added to 50-ml Erlenmeyer flasks. Deionized water (10 ml) was added to each flask and the slurry buffered with 2% (w/v) calcium carbonate. The flasks were sealed with serum stoppers (Suba-Seal, UK). If subsequent aqueous additions of nitrogen compounds were to be made, the initial volume of deionized water was reduced accordingly. The flasks were incubated on a gyratory shaker (200–250 rev min^{-1}) at 30 °C. Where desired, the initial gas phase concentration of CH_4 and C_2H_2 was established by syringe injection after removing an equivalent volume of the gas phase. During preincubation with methane the gas phase concentrations of CH_4 and O_2 were re-established every 2 days by evacuation and refilling with air and injection of methane. If the oxygen concentration was to be adjusted, pure O_2 was added by syringe injection. Moisture loss was compensated by the addition of an equivalent weight of deionized water.

The data presented are the means of duplicate flasks.

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Analyses. At desired intervals, 0.5 ml of the gas phase was removed using a 1-ml disposable glass syringe equipped with a Mininert valve (Precision Sampling Corp., Baton Rouge, LA.). The samples were analysed for O_2 and CH_4 by a single injection into a split column gas chromatograph equipped with a 174 cm×3.3 mm OD column of molecular sieve 5A connected to a thermal conductivity detector and a 200 cm×3.3 mm OD column of Porapak N connected to a flame ionization detector. Analysis of CO_2 was as described by Brouzes et al. (1971). The data were corrected for gases dissolved in the aqueous phase (Wilhelm et al. 1977) and for leakage as determined by flasks containing the appropriate initial volume of gas(es) but without the soil.

Pure culture study. Methylosinus trichosporium OB3b (obtained from R. Whittenbury via. T. Yoshinari) was used as a reference culture to compare the stoichiometry of the conversion of methane to carbon dioxide. Cells were grown in stoppered 500-ml Erlenmeyer flasks containing 100 ml modified ammonium mineral salts (AMS) medium (Whittenbury et al. 1970). The $FeSO_4$ was replaced with Sequestrene NaFe (CIBA Geigy Chemicals, 13% Fe) and the $CuCl_2 \cdot 2H_2O$ in the trace element solution replaced with $CuSO_4 \cdot 5H_2O$. The gas phase (30 kPa CH_4) was established by partial evacuation with a vacuum pump and replenishing the head space to 1 atm with pure filter-sterilized CH_4.

Flasks were incubated at 30 °C on a gyratory shaker (200–250 rev min^{-1}). The cells were harvested in the late-log phase by centrifugation at 16000 xg for 12 min at 4 °C. The cells were washed 3 times and resuspended in AMS medium to a density of 5.4×10^9 cells ml^{-1}. An 8-ml aliquot of this cell suspension was added to each of a series of 60-ml serum bottles each containing 2 ml AMS. The bottles were sealed with grey butyl rubber stoppers (Wheaton Scientific, Millville, NJ) and filter-sterilized CH_4 added by syringe injection after withdrawing an equivalent volume of the gas phase. The bottles were incubated under the conditions described above.

Results

The soil used in this study contained populations of both methanogens and methanotrophs. Under anaerobic conditions in the presence of endogenous ammonium and nitrate, acetylene-sensitive methane production was observed after a lag of about 4 days (Fig. 1). The delay was likely due to the gradual lowering of redox potential, the relief of nitrate inhibition after reduction or denitrification of the nitrate or to both of these factors. The sensitivity of methanogens to acetylene and nitrate has been previously reported (Chen et al. 1972; Raimbault 1975; Knowles 1979).

Soil which was not previously exposed to exogenous methane consumed added methane at rates (0–8 h) of 3.7 and 1.7 µmol CH_4 $flask^{-1}$ h^{-1} in the presence of 10 m*M* NH_4Cl and 10 m*M* KNO_3, respectively (data not shown). After preincubation with 10 m*M* NH_4Cl under 20 kPa CH_4 (1 kPa = 16.1 µmol $flask^{-1}$) for 6 days the soil slurry consumed 254 µmol CH_4 $flask^{-1}$ within 13 h (Fig. 2). The initial (0–2 h) rate of consumption was 40.2 µmol CH_4 $flask^{-1}$ h^{-1}, indicating enrichment of the methane oxidizers.

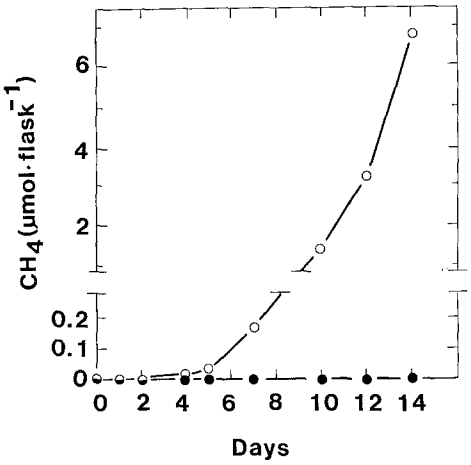

Fig. 1. Production of methane by the humisol under anaerobic conditions in the presence (●) and absence (○) of 5 kPa C_2H_2

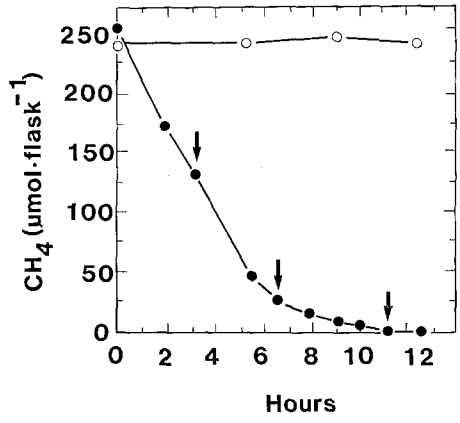

Fig. 2. Oxidation of methane by the humisol under aerobic conditions. Control flasks were incubated without soil (○). Slurries were preincubated for 6 days with 10 m*M* NH_4Cl under 20 kPa CH_4 in air and then regassed at zero time. Oxygen was added at times indicated by the *arrows*

Methane oxidation activity

The methane oxidizers dominated within 2 days of incubation in the presence of 20 kPa CH_4, as measured by the rates of oxygen consumption and production of carbon dioxide (Table 1). After 10 days of incubation, the rates of oxygen consumption and carbon dioxide production were 98.1% and 72.1% higher, respectively, in the presence of methane than in its absence. The patterns of net gas consumption and production showed that equimolar amounts of oxygen and methane were consumed (Fig. 3). In addition, the molar yield of carbon dioxide from methane was low, indicating that a large fraction of the carbon was being incorporated into cell biomass.

In order to define more precisely the yield of CO_2 from methane, the soils were preincubated for 8 days with 20 kPa CH_4. The initial (0–5.6 h) rates of CH_4

Table 1. Rates of oxygen consumption and production of carbon dioxide in humisol slurries in the presence and absence of methane[a]

Gas phase	N source	ΔO_2 (μmol flask^{-1} h^{-1})		ΔCO_2 (μmol flask^{-1} h^{-1})	
		Days		Days	
		2	10	2	10
Air	Endogenous	1.3±0.3	1.1±0.1	1.7±0.6	6.4±0.3
Air	NH_4Cl (10 m*M*)	2.1±0.1	0.9±0.1	2.5±0.0	5.8±0.1
Air/CH_4 (20 kPa)	NH_4Cl (10 m*M*)	13.9±0.1	52.8±0.6	5.2±0.1	21.9±0.6

[a] Values are means of duplicate flasks ± SE

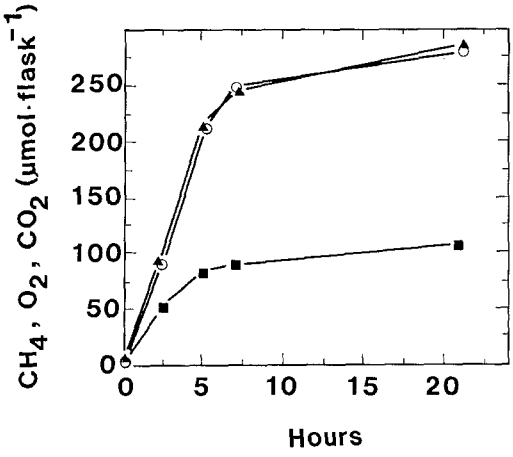

Fig. 3. Total consumption of methane (○) and oxygen (▲) and production of carbon dioxide (■) by the humisol after 10 days of preincubation under 20 kPa CH_4. Initial concentrations of CH_4 and O_2 were 333 and 296 μmol flask^{-1}, respectively

consumption and CO_2 production were then measured and compared with those of a pure culture of *Methylosinus trichosporium* OB3b, a typical type II methanotroph incorporating carbon via the serine pathway. After correcting for carbon dioxide produced in the absence of methane, the soil showed 0.27 mol CO_2 produced/mol CH_4 consumed (Table 2). This result compared favourably to the 0.20 ratio observed with *M. trichosporium* OB3b (Table 2).

Kinetics of methane oxidation

The effects of methane and oxygen concentrations on methane oxidation were studied in soils which were pre-enriched with 20 kPa CH_4 for 6 days. The kinetic constants were derived from double reciprocal plots of gas consumption rates (0–1.5 h) and concentrations of gas in solution. Methane and oxygen were maintained at dissolved concentrations of greater than 150 μ*M* and 200 μ*M*, respectively, during the experiment in which the effect of the particular gas on methane oxidation was not under investigation.

Table 2. Stoichiometry of conversion of methane to carbon dioxide by *Methylosinus trichosporium* OB3b and humisol slurries[a]

	ΔCH_4 (μmol flask^{-1})	ΔCO_2 (μmol flask^{-1})	CO_2/CH_4
Methylosinus trichosporium OB3b	51.0±0.9	10.3±0.3	0.20
Soil	190.5±10.4	50.4±7.1	0.27

[a] Culture and soil incubated in the presence of 10 m*M* NH_4Cl for 7.8 and 5.6 h, respectively. Soil was preincubated with 20 kPa CH_4 for 8 days prior to assay. Initial CH_4 concentrations were 63 and 313 μmol flask^{-1} for the culture and soil, respectively. Carbon dioxide produced from CH_4-independent activity in soil is not included in the calculation. Cells were added to give an initial protein concentration of 66 μg ml^{-1}. Data are means of duplicate flasks ±SE

The rates of methane oxidation showed typical Michaelis-Menten kinetics (Fig. 4). The values of V_{max} and apparent K_m for methane were 51.5 μmol flask^{-1} h^{-1} and 66.2 μ*M*, respectively. A least squares analysis of the double reciprocal plot showed a correlation coefficient of 0.991.

The rates of methane-dependent oxygen consumption were significantly reduced at dissolved oxygen concentrations below 58 μ*M* and completely inhibited at 9 μ*M* (Fig. 5). The double reciprocal plot of rate and oxygen concentration showed typical sigmoid kinetics (Fig. 5, open circles), suggesting positive homotropic cooperativity between oxygen and methane. However, in a type II methanotrophic bacterium (strain OU-4-1), a random bireactant mechanism was described for CH_4 and O_2 in which the binding of one substrate decreased the affinity for the other (Joergensen 1985). Replotting the present data as $(1/V)\times(1/S^2)$ achieved linearity ($r = 0.996$) and is consistent with the pattern exhibited by enzymes which follow sigmoid kinetics (Laidler and Bunting 1973). The values of V_{max} and apparent K_m for oxygen derived from the latter plot were 51.3 μmol flask^{-1} h^{-1} and 37.0 μ*M*, respectively.

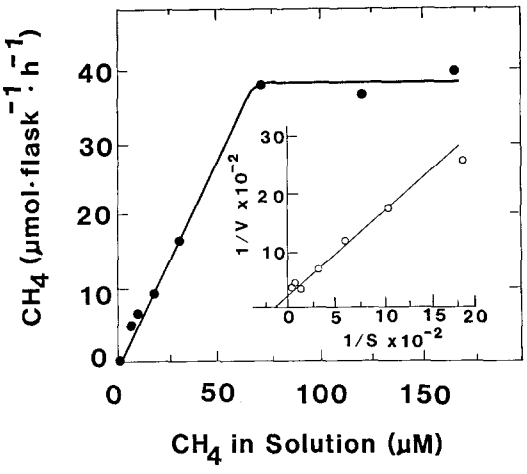

Fig. 4. Rates of methane oxidation at different concentrations of dissolved methane. The humisol was preincubated for 6 days under 20 kPa CH_4. The *inset* shows a double reciprocal plot of the data

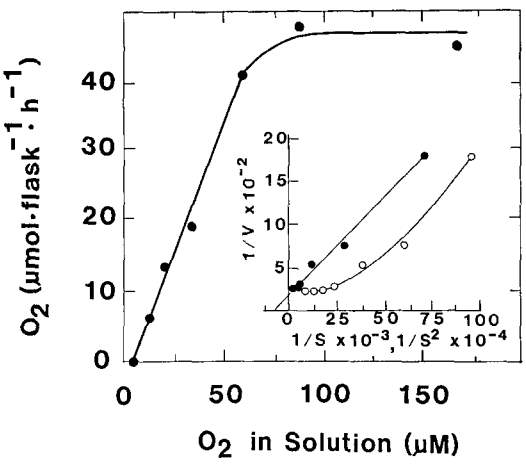

Fig. 5. Rates of methane-dependent oxygen consumption at different concentrations of dissolved oxygen. The humisol was preincubated for 6 days under 20 kPa CH_4. The *inset* shows double reciprocal plots of 1/S (○) and $1/S^2$ (●) versus 1/V

Discussion

Methane oxidation activity

Methane is oxidized to carbon dioxide by a series of two-electron oxidation steps via methanol, formaldehyde and formate (Anthony 1982). Carbon is assimilated either at the level of formaldehyde or as a combination of formaldehyde and carbon dioxide. The stoichiometry of methane oxidation in a humisol showed that 0.27 mol CO_2 were produced and 1.0 mol O_2 consumed/mol CH_4 oxidized. The molar ratio of CO_2 produced from CH_4 oxidation by *M. trichosporium* OB3b in this study was 0.20. Whittenbury et al. (1970) also described CO_2 yields of 0.2–0.3 mol at the expense of 1.0–1.1 mol O_2 and 1.0 mol CH_4 in pure cultures of obligate methanotrophs. Therefore, the results from both the humisol and pure culture studies imply that 70%–80% of the oxidized methane was incorporated into cell material.

An examination of methane carbon assimilation and CO_2 production in freshwater systems showed cellular incorporation to be only 25%–30% (Rudd and Hamilton 1975; Harrits and Hanson 1980) although values as high as 60% have been reported (Panganiban et al. 1979). The higher molar yields of carbon dioxide per mole of methane in these studies compared with the present study likely reflect differences in the types of methane oxidizers and/or their physiological states as described by Whittenbury et al. (1970). In a marine system, >98% of the methane carbon was recovered as CO_2, leading to the conclusion that methane was being used primarily as an energy source (Griffiths et al. 1982). The authors further suggested that the methane oxidizers were either heterotrophs using organic compounds or autotrophs using CO_2 for their carbon requirements. The relatively low CO_2 yields exhibited in our study strongly indicate that methane oxidation in the soil was being catalysed by obligate methanotrophs.

Kinetics of methane oxidation

The apparent K_m for methane in the soil was 66 μ*M*, which compares favourably with other kinetic studies using whole cell cultures of methane oxidizers. Typical values reported in the literature are 60 μ*M* (Takano and Terui 1975), 32–44 μ*M* (Linton and Buckee 1977) and 45–48 μ*M* (O'Neill and Wilkinson 1977). The purified methane monoxygenase (MMO) of the type II obligate methanotroph *Methylosinus trichosporium* OB3B has a K_m for methane of 66 μ*M* (Anthony 1982). The soluble MMO of *Methylococcus capsulatus* (Bath) has a K_m of 160 μ*M* (Colby et al. 1977). Other investigators have found values as low as 1–4 μ*M* in pure culture studies (Nagai et al. 1973; Lamb and Garver 1980; Joergensen and Degn 1983), 5 μ*M* in lake water (Rudd and Hamilton 1975), and 10 μ*M* in sediments (Lidstrom and Somers 1984).

The kinetics of methane oxidation in soil are of interest since it has been reported that some soils can act as net sinks of atmospheric methane (Harriss et al. 1982; Keller et al. 1983). According to Conrad (1984), however, the reported K_m values for methane in aquatic and pure culture systems are too high to support the growth of methane oxidizers on the trace levels of gases which would be found in the aqueous phase of soils (ca. 2.5 n*M* at 20 °C).

The present study is the first to report Michaelis-Menten kinetics for methane oxidation in a soil system. The results are in general agreement with previous reports that the affinities of methane oxidizers for methane are low with respect to fixing atmospheric

methane. The data may not be truly representative of in situ conditions since the kinetic constant was defined in pre-enriched soils and the measurements of gases dissolved in the liquid phase were based on the gas phase concentrations. However, the soil used in this study also showed detectable activity of methanogens under anaerobic conditions. Therefore, the methane oxidizers in this particular soil system may not be entirely dependent on atmospheric methane for growth and survival. Alternatively, if the methane oxidizers are able to couple the oxidation of ammonia to energy generation and utilization (Malashenko et al. 1979), the growth on trace levels of methane would not be predicted from the affinity of their enzymes for methane. The ability of methane-oxidizers isolated from these soils to co-oxidize ammonium is currently being studied.

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