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High-Affinity Methane Oxidation by a Soil Enrichment Culture Containing a Type II Methanotroph

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Methanotrophic bacteria in an organic soil were enriched on gaseous mixing ratios of <275 parts per million of volume (ppmv) of methane (CH_4). After 4 years of growth and periodic dilution (>10^{20} times the initial soil inoculum), a mixed culture was obtained which displayed an apparent half-saturation constant [$K_{m(app)}$] for CH_4 of 56 to 186 nM (40 to 132 ppmv). This value was the same as that measured in the soil itself and about 1 order of magnitude lower than reported values for pure cultures of methane oxidizers. However, the $K_{m(app)}$ increased when the culture was transferred to higher mixing ratios of CH_4 (1,000 ppmv, or 1%). Denaturing gradient gel electrophoresis of the enrichment grown on <275 ppmv of CH_4 revealed a single gene product of *pmoA*, which codes for a subunit of particulate methane monooxygenase. This suggested that only one methanotroph species was present. This organism was isolated from a sample of the enrichment culture grown on 1% CH_4 and phylogenetically positioned based on its 16S rRNA, *pmoA*, and *mxaF* gene sequences as a type II strain of the *Methylocystis*/*Methylosinus* group. A coculture of this strain with a *Variovorax* sp., when grown on <275 ppmv of CH_4, had a $K_{m(app)}$ (129 to 188 nM) similar to that of the initial enrichment culture. The data suggest that the affinity of methanotrophic bacteria for CH_4 varies with growth conditions and that the oxidation of atmospheric CH_4 observed in this soil is carried out by type II methanotrophic bacteria which are similar to characterized species.

Methane-oxidizing bacteria inhabit the aerobic interfaces of methanogenic environments and reduce the potential methane (CH_4) emissions from these environments (7, 15, 30). Atmospheric CH_4, which has a present mixing ratio of 1.7 parts per million of volume (ppmv), is also oxidized microbially in aerobic upland soils (15). This process represents about 10% of the atmospheric CH_4 sink (10).

The identity of these atmospheric-CH_4 oxidizers is unknown. Whereas soil CH_4 oxidation rates can remain steady for >4 months at 1.7 ppmv of CH_4 (34), calculations based on the kinetic constants of known methanotrophic species suggest that these organisms are incapable of such extended survival (6). Atmospheric CH_4 should not supply sufficient cellular maintenance energy plus reducing power for the methane monooxygenase (MMO) enzyme. Studies with *Methylosinus trichosporium* and *Methylobacter albus* (*Methylomicrobium album*) seem to confirm this (32, 34).

However, the kinetic properties of CH_4 oxidation in upland soils are different from those in pure methanotroph cultures. Apparent half-saturation constants [$K_{m(app)}$] of various type I and II methanotrophs range from 0.8 to 66 μM CH_4 (5, 18). While some of these values are overestimated due to diffusion limitation, a lower limit of 0.8 to 2 μM is probable (18). Environmental samples from the aerobic interfaces of methanogenic habitats, such as lake sediments and landfill cover soils, have $K_{m(app)}$ values in the same range (7, 39). However, the $K_{m(app)}$ values for upland forest and agricultural soils are 1 to 3 orders of magnitude lower, at 10 to 280 nM CH_4 (1, 3, 7, 8, 13). Although a lower-affinity activity can be induced by enrichment with atmospheres containing 10% CH_4 (1), the methanotrophs normally active in these soils seem to be adapted to reduced CH_4 levels. Either uncharacterized species are involved in atmospheric-CH_4 oxidation or unknown physiological changes are induced in known methanotrophic species living in these soils.

The $K_{m(app)}$ for CH_4 consumption in an organic soil from Ottawa, Canada, was estimated as 80 to 90 nM (8). This is in the same order of magnitude as values measured in other aerobic upland soils, although slightly higher. Values as low as 10 nM have been measured in soils (1, 3, 7, 13). Here we report on experiments aimed at enriching and characterizing the organisms responsible for the high-affinity activity in this organic soil.

MATERIALS AND METHODS

Sampling site. The study site has been described previously (8, 9). It is an organic (60% combustible matter), neutral (pH 6.7 to 7.2) soil located on the Central Experimental Farm of Agriculture and Agri-Food Canada in Ottawa. The soil was sampled from a depth of 5 to 20 cm in August 1993.

Enrichment of soil with <275 ppmv of CH_4. Enrichment cultures were made in nitrate mineral salts medium (NMS) (14) containing 3 nM Cu and 1 mM phosphate buffer at pH 6.0. Deionized distilled or twice-distilled water was used. Initially, 0.15 g of soil was added to 10 ml of NMS in 125-ml serum vials. The vials were capped with autoclaved butyl rubber stoppers, and CH_4 was added at a final gaseous mixing ratio of 75 ppmv. The enrichment cultures were incubated at 25°C. The CH_4 was replaced after declining to below 25 ppmv. After 7 months, and periodically thereafter for 4 years, subsamples of the enrichment culture were transferred into fresh medium. The CH_4 mixing ratio in the vials varied considerably during this enrichment period. It declined to 1 to 50 ppmv of CH_4 before being replaced but never exceeded 275 ppmv.

Some modifications were made during the enrichment period. Two years into the enrichments and thereafter, a pH 6.8 buffer and a 10-times-strength trace element solution (30 nM Cu) were used in the NMS medium. Since the butyl rubber stoppers often exuded inhibitory compounds after being autoclaved, the stoppers used after the initial transfer were sterilized by washing them in ethanol (50 to 80% [vol/vol]) followed by rinsing them three times in sterile distilled water.

All experiments described below were performed after the initial 4-year enrichment period. During this time, the culture had been diluted to >10^{20} times

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TABLE 1. $K_{m(app)}$ values measured for methanotrophic bacteria, methanotrophic enrichment cultures, and soil[a]

Sample (reference)	$K_{m(app)}$ (μM CH_4)	
	<1 μM	>1 μM
Literature		
Methanotrophs, MMO (5, 18)		0.8–66
Aerobic interfaces of methanogenic soil (7, 39)		1.0–11
Aerobic upland soils (1, 3, 7, 8, 13)	0.010–0.280	
Aerobic upland soils, enriched with 20% CH_4 (1)	0.015–0.450	1.7–28
Organic soil (8)	0.060–0.280[b]	
Organic soil, enriched with 10% CH_4 (8a)		2.7–6.7
This study		
Enrichment culture		
Grown on <275 ppmv of CH_4	0.056, 0.083, 0.173, 0.186	
Transferred to 1,000 ppmv of CH_4	0.330, 0.566	
Transferred to 1% CH_4		1.96, 2.60
Coculture; type II methanotroph LR1 + *Variovorax* sp.	0.129, 0.177, 0.183, 0.188	

[a] For the literature, the range of reported values is shown. For experimental data, values from at least two trials (each with a separately grown culture) are shown.
[b] Estimated as 80 to 90 nM in the absence of NH_4^+.

the initial soil inoculum. Within this period a serial dilution series had been performed, and only the highest positive dilution (10^7 times) was maintained.

Kinetic experiments. Half-liter batches of the culture were grown on <200 ppmv of CH_4 for 1 to 4 months. Since our aim was to study substrate affinity rather than specific activity, cell counts were not done. However, from the dilution series described in the previous section, methanotroph densities of $<10^7$ cells ml^{-1} could be expected after such incubation times. Aliquots (2 to 8 ml, depending on the experiment) of the culture were transferred to 14-ml serum vials, and chloramphenicol at a final concentration of 50 μg $liter^{-1}$ was added to prevent further enzyme production. The vials were capped with butyl rubber stoppers and injected with CH_4 at mixing ratios ranging from 5 to 1,000 ppmv. The vials were incubated at 25°C and rotated around the short axis at 21 rotations per min. At 1- to 2-day intervals for 3 to 10 days, CH_4 was measured by injection of 0.3-ml gas samples into a Carlo Erba gas chromatograph equipped with a flame ionization detector (oven temperature, 100°C; injector temperature, 140°C; 3-m by 3-mm Porapak Q column).

Methane oxidation rates were estimated by linear regressions of CH_4 mixing ratios versus time. The regressions were effectively linear at CH_4 levels greater than the $K_{m(app)}$ (r^2, usually >0.95), proving that initial rates were being measured. The maximum decline of CH_4 over the incubations was always <50%. At each CH_4 concentration, blank vials containing only water were included to estimate CH_4 removal during gas chromatographic sampling and to correct the CH_4 oxidation rates in the culture. These corrected rates were plotted against the CH_4 levels at the time midpoint and fitted to a Michaelis-Menton hyperbolic model by using the least-squares iterative fitting procedure of Origin 4.1 (Microcal Software, Inc., Northampton, Maine).

Transfer of the enrichment culture into CH_4 at higher mixing ratios. The enrichment culture was inoculated into 50 ml of NMS in 125-ml serum vials and grown on CH_4 at initial mixing ratios of 1,000 ppmv and 1%. The CH_4 was replaced after declining to <20% of these initial values. The CH_4 was replaced five times for the 1,000-ppmv treatment and six times for the 1% treatment. Kinetic experiments were then performed as described above at a range of 20 to 3,000 ppmv of CH_4 (for the 1,000-ppmv enrichment culture, 1-ml aliquots were diluted to 2 ml and measured at 2-day intervals for 6 days; for the 1% enrichment culture, 1-ml aliquots were diluted to 6 ml total and measured at 1 or 2 intervals of 3 h).

Isolations. Members of the low-CH_4 enrichment culture (<275 ppmv of CH_4) were isolated by performing a decimal dilution series in liquid NMS medium and making spread plates of each dilution onto NMS medium solidified with 15 g of Bacto Agar (Difco Laboratories, Detroit, Mich.) $liter^{-1}$. The plates were incubated at 25°C in closed chambers containing a gaseous mixing ratio of 3% CH_4. Colonies which formed on plates of the highest two dilutions showing growth (10^5 to 10^6 times the initial inoculum) were restreaked for isolation (i) onto NMS solidified with Noble agar (Difco) and incubated under 3% CH_4 and (ii) onto plates of a general medium for culturing nonfastidious organisms, R2A agar (Difco), and incubated under air. Because we failed to isolate a methanotroph in this way (see Results), the procedure was repeated with a sample of the enrichment culture grown on 1% CH_4 (see the previous section).

DNA extraction. Extraction of DNA from the low-CH_4 enrichment culture (<275 ppmv of CH_4) and from the various isolates was adapted from the procedure of Moré et al. (28). Approximately 1 g of sterilized (170°C for 4 h) zirconia-silica beads of 0.1-mm diameter (Biospec Products Inc., Bartlesville, Okla.) was added to cell pellets in 2-ml screw-cap tubes. The cells and beads were suspended by vortexing them in 800 μl of NaH_2PO_4-Na_2HPO_4 buffer (120 mM; pH 8) and 260 μl of sodium dodecyl sulfate solution (10% [wt/vol] sodium dodecyl sulfate, 0.1 M NaCl, 0.5 M Tris-HCl, pH 8). The cells were lysed in a bead beater (Fastprep FP120; Savant Instruments Inc., Farmingdale, N.Y.) at a setting of 6.5 m s^{-1} for 45 s. The tubes were centrifuged for 3 min at 12,000 × *g*, and the supernatant was collected. The beads were then resuspended in 700 μl of NaH_2PO_4-Na_2HPO_4 buffer, and the DNA extraction was repeated. Proteins and debris were precipitated from the supernatant by adding 2.5 volumes of 7.5 M ammonium acetate, incubating the solution for 5 min on ice, and centrifuging it at 12,000 × *g* for 3 min. The DNA was precipitated by centrifugation (4°C; 45 min; 12,000 × *g*) with 70% (vol/vol) isopropanol added. The DNA pellet was washed with 70% (vol/vol) ethanol at 4°C, dried, and resuspended in 200 μl of elution buffer (Bio-Rad, Munich, Germany).

Comparative 16S rRNA gene sequence analysis. PCR-mediated amplification of the 16S rRNA genes from positions 28 to 1491 (numbering according to the International Union of Biochemistry nomenclature for *Escherichia coli* 16S rRNA) and sequencing analyses were done as previously described (22). Phylogenetic placement of strain LR1 was performed with the ARB program package (37). The 16S ribosomal DNA (rDNA) sequence of strain LR1 was integrated into a database of about 6,000 complete or partial bacterial 16S rRNA sequences (24, 31, 38) with the automatic alignment tool of the ARB program package. This procedure showed strain LR1 to be a member of the alpha subclass of the class *Proteobacteria*. The phylogenetic position of strain LR1 was determined in more detail by comparing its 16S rDNA gene sequence with alpha proteobacterial reference sequences. The tree topology was evaluated by a distance matrix analysis. For phylogenetic inference, the only nucleotide sequence positions considered were those which contained identical nucleotides in at least 50% of a representative selection of 16S rRNA sequences from the major lineages of the alpha subclass of proteobacteria (1,353 nucleotide sequence positions). Evolutionary distance values between pairs of microorganisms were calculated with the Felsenstein correction (ARB) (11). The tree was constructed by the neighbor-joining algorithm (33). The statistical significance values of interior branch points were tested in a bootstrap analysis by the neighbor-joining method (ARB; 1,000 data resamplings).

Comparative analysis of *pmoA* and *mxaF* gene fragments. (i) PCR amplification. Primers targeting gene fragments of *pmoA* (A189f and A682r) (16), coding for a subunit of particulate MMO (pMMO), and of *mxaF* (*mxaF* f1003 and *mxaF* r1561) (25, 27), coding for the large subunit of methanol dehydrogenase, were used for PCR amplification. A GC clamp (CCCCCCCCCCCCCCGCCCCCCGC CCCCCGCCCCCGCCGCCC) was attached to the 5′ end of each forward primer. PCR was performed in 50-μl reaction mixtures with an Eppendorf (Hamburg, Germany) gradient cycler. *Taq* polymerase (Perkin-Elmer Applied Biosystems, Branchburg, N.J.) was added to 0.5 μM concentrations of each primer and a PCR premix (Epicentre Technologies, Madison, Wis.). The *mxaF* fragment was amplified as described previously (27). For the *pmoA* amplification a touchdown program was developed, consisting of an initial denaturing step (94°C; 3 min) followed by 20 touchdown cycles (62 to 52°C), eight further cycles (52°C for 1 min followed by 72°C for 45 s), and a final extension (72°C; 5 min). The PCR products were analyzed on 3% agarose gels stained with ethidium bromide.

(ii) DGGE. PCR products were separated on 1-mm-thick polyacrylamide gels (6.5% [wt/vol] 37.5:5 acrylamide-bisacrylamide) (Bio-Rad) poured on GelBond support medium (FMC Bio Products, Rockland, Maine). They were prepared and run in Tris-acetic acid-EDTA buffer (0.04 M Tris-base, 0.02 M sodium acetate, 1 mM EDTA) at pH 7.4 and 60°C. The Dcode electrophoresis system (Bio-Rad) was used for separation. Denaturing gradient gel electrophoresis (DGGE) conditions for the various PCR products were optimized by perpen-

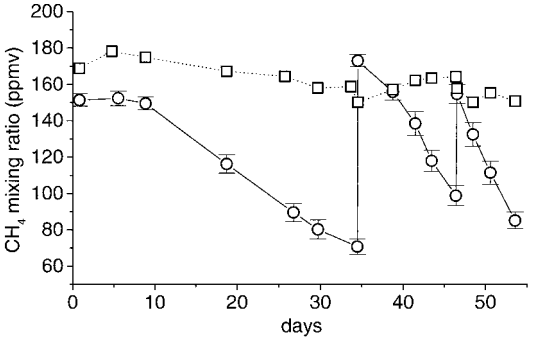

FIG. 1. Oxidation of CH_4 at mixing ratios of <200 ppmv (○) in closed vials (125 ml) containing 50 ml of NMS inoculated with the enrichment culture at day 0. Methane was added to the vials at 34 and 46 days. The data are the means of results with six vials ± 1 standard error of the mean. □, mean of results with three uninoculated vials.

dicular DGGE. For the *pmoA* fragments, a gradient of 35 to 80% denaturant (80% corresponded to 6.5% [vol/vol] acrylamide, 5.6 M urea, and 32% [vol/vol] deionized formamide) at a constant voltage of 200 V for 6 h was used. For the *mxaF* fragments, a gradient of 20 to 70% denaturant at a constant voltage of 150 V for 5 h was used. The gels were stained with 1:50,000 (vol/vol) SYBR-green I (Biozym, Hessisch-Oldendorf, Germany) for 30 min and then scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

(iii) DNA sequencing and construction of *pmoA*- and *mxaF*-based trees. Distinct bands were excised from the SYBR-green-stained gels with sterile pipette tips and suspended in 200 μl of H_2O. The bands were reamplified and rerun on DGGE to ensure purity.

The PCR products from the excised bands were purified with the Easy-Pure DNA purification kit (Biozym). The concentration and purity of the PCR products were determined by absorption at 260 and 280 nm of a 1:20 dilution. The sequencing reactions were performed in both directions with the PRISM dye terminator cycle-sequencing kit (Perkin-Elmer Applied Biosystems). Products of the cycle-sequencing reaction were purified from excess dye terminators and primers with Microspin G-50 columns (Pharmacia, Uppsala, Sweden) and sequenced on an automatic DNA sequencer (model 373A; Perkin-Elmer Applied Biosystems). Phylogenetic trees were constructed with the cluster alignment algorithm of the DNA-Star software package (Lasergene Inc., Madison, Wis.).

Comparative sequence analysis of *mmoX*. A gene fragment of *mmoX* coding for the alpha subunit of soluble MMO (sMMO) was amplified from both the low-CH_4 enrichment culture (<275 ppmv of CH_4) and the methanotrophic isolate LR1 with primers (*mmoX* f882 and *mmoX* r1403) under PCR conditions described previously (25). The resulting PCR products were checked for size and purity on a 1.5% agarose gel and purified with the Prep-A-Gene system (Bio-Rad). Sequencing was performed as described above.

Nucleotide sequence accession numbers. The nucleotide sequences of the nearly complete 16S rRNA gene and of partial *mmoX*, *mxaF*, and *pmoA* genes and gene fragments of strain LR1 have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. Y18442, Y18440, Y18441, and Y18443, respectively.

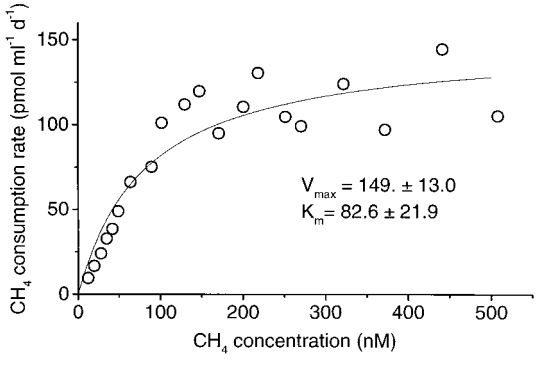

FIG. 2. Kinetic curve of CH_4 oxidation in a soil enrichment culture which was grown continuously on CH_4 at a gaseous mixing ratio of <275 ppmv. Each symbol represents the mean of results with two samples.

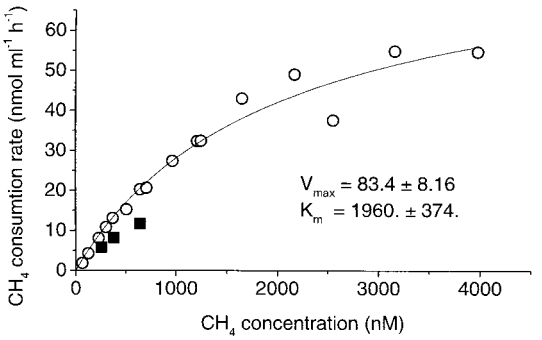

FIG. 3. Kinetic curve of CH_4 oxidation in a soil enrichment culture initially grown on CH_4 at a gaseous mixing ratio of <275 ppmv and later on 1% CH_4. Each symbol represents the mean of results with two samples. ■, samples diluted 1:1 with water.

RESULTS

Soil enrichment cultures grown on <275 ppmv of CH_4. Soil enrichment cultures grown on 10 to 20% CH_4 display low-affinity kinetics [$K_{m(app)} > 1$ μM (Table 1)]. We therefore chose a lower CH_4 mixing ratio in an attempt to enrich for higher-affinity methanotrophic activity.

The CH_4 oxidation rate of soil inoculated into NMS showed a gradual increase with time when the soil was incubated at CH_4 mixing ratios of as little as 75 ppmv. An enrichment culture was obtained by continuous growth on <275 ppmv of CH_4 for 4 years. The increase in activity was very slow, typically requiring a week or more to double. An example showing the increasing rate of CH_4 oxidation over time in the enrichment culture is shown in Fig. 1. Blank vials containing uninoculated medium plus CH_4, which were sampled in exactly the same way as the enrichment cultures, never developed methanotrophic activity. Therefore, contamination of the cultures with a methanotroph from another source was unlikely.

Kinetics. A typical kinetic curve from the low-CH_4 enrichment culture is shown in Fig. 2. The measured $K_{m(app)}$ in four trials varied from 56 to 186 nM CH_4 (40- to 132-ppmv mixing ratio) (Table 1). This range overlaps the $K_{m(app)}$ estimated for the organic soil itself (Table 1).

When samples of the enrichment were transferred to new medium and grown on CH_4 at higher mixing ratios, the $K_{m(app)}$ values increased (Table 1). Growth on 1,000 ppmv of CH_4 resulted in $K_{m(app)}$ values higher than those in the initial low-CH_4 enrichment (<275 ppmv of CH_4) but still lower than those in pure methanotroph cultures. Enrichments grown on 1% CH_4 had $K_{m(app)}$ values typical for methanotrophic cultures (Table 1). A kinetic curve for a culture grown on 1% CH_4 is shown in Fig. 3. The high $K_{m(app)}$ value was not a result of phase transfer limitation or poor mixing of CH_4, as demonstrated by the fact that diluting the culture 1:1 with water resulted in about a 50% reduction of the CH_4 oxidation rate (Fig. 3).

Members of the methanotrophic enrichment culture. When samples of the low-CH_4 enrichment culture (<275 ppmv of CH_4) were plated onto NMS agar, various types of colonies grew. These had sizes and morphologies typical of heterotrophic organisms which grow on trace contaminants in the medium (14). Four separate organisms which grew when transferred to complex medium (R2A agar) were isolated. These were identified based on partial 16S rRNA gene sequences. The closest relatives of these isolates were "*Pseudomonas pavonacea*" IAM1155 (similarity, 99.0% based on an analyzed

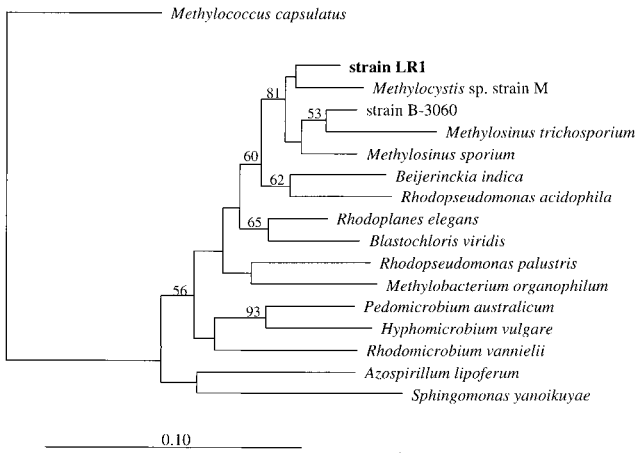

FIG. 4. Phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship of isolate LR1 to other type II methanotrophs of the *Methylocystis*/*Methylosinus* group and to representative members of the alpha subclass of the class *Proteobacteria*. The 16S rRNA gene sequence from *Methylococcus capsulatus* was used as an outgroup reference. The numbers indicate the bootstrap values (percentage of outcome) for the respective interior branch points from a neighbor-joining test. Only values above a threshold of 50% are shown. The scale bar represents the estimated number of base changes per nucleotide sequence position.

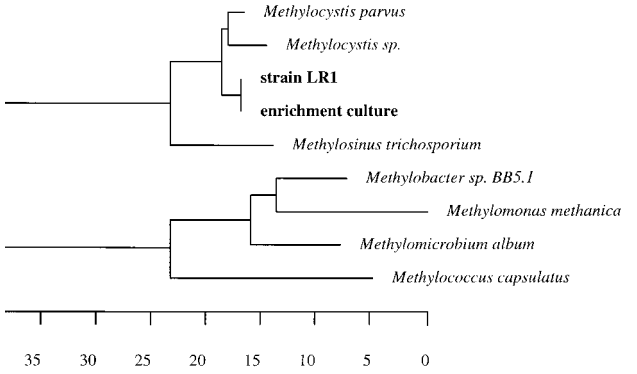

FIG. 5. Unrooted phylogenetic tree, based on derived amino acid sequences of *pmoA* gene fragments, showing the identity of the *pmoA* product from isolate LR1 and that from the high-affinity enrichment culture (grown on <275 ppmv of CH_4) and their relationship to other methanotrophic species. The distance bar represents percent dissimilarity.

stretch corresponding to *E. coli* 16S rRNA numbering positions 104 to 1293), *Variovorax paradoxus* (similarity, 99.2%; numbering positions 158 to 1192), *Bradyrhizobium elkanii* USDA76 (similarity, 98.5%; numbering positions 28 to 819), and *Hyphomicrobium vulgare* MC-750 (similarity, 96.9%; numbering positions 28 to 908).

None of these isolates consumed CH_4 in pure culture, and none showed a *pmoA* gene product (data not shown). However, transfer of the low-CH_4 (<275 ppmv of CH_4) liquid enrichment culture to an atmosphere containing 1% CH_4 resulted in a turbid culture which did produce thick methanotroph colonies (14) when streaked onto NMS agar and grown on 3% CH_4. Isolated colonies of a methanotroph designated LR1 were selected and stored in liquid NMS on 1% CH_4.

Gene primers for *mmoX* (coding for the alpha subunit of sMMO) and *pmoA* (coding for a subunit of pMMO) both gave a signal from isolate LR1. The membership of strain LR1 within the type II methanotrophs of the *Methylocystis*/*Methylosinus* cluster in the alpha subclass of the class *Proteobacteria* was clearly shown by comparative analysis of its 16S rRNA (Fig. 4), its *pmoA* (Fig. 5), and its *mxaF* (Fig. 6) gene sequences.

DGGE analyses. The results of the DGGE analyses suggested that there was a single methanotrophic species present in the low-CH_4 enrichment culture (<275 ppmv of CH_4). The *pmoA* primer system, which should detect all known methanotrophic species, produced a single band on the DGGE for the enrichment (Fig. 7). Although methanotrophs appear to contain two copies of *pmoA* (36), these are not always distinguishable on DGGE. Two *pmoA* bands were occasionally evident from methanotroph isolates when DGGE gels were silver stained, but only a single band was evident from the enrichment culture and from isolate LR1 (data not shown).

The *mxaF* primer system also produced only one clear band (Fig. 7). A second faint band may have been present in the *mxaF* gel, but is migration pattern corresponded to that of a methylotrophic *Hyphomicrobium* sp. isolated from the culture.

The sequences of the *pmoA* (Fig. 5), *mxaF* (Fig. 6), and *mmoX* (data not shown) PCR products retrieved from the low-CH_4 enrichment (grown on <275 ppmv of CH_4) were all identical to those of the respective PCR products retrieved from isolate LR1.

Isolate LR1 was therefore the only detectable methanotroph in the enrichment culture grown on <275 ppmv of CH_4. The data cannot completely rule out the possibility that another methanotroph was present, perhaps in much lower abundance, but its *mxaF*, *pmoA*, or *mmoX* genes were not detected. They also cannot rule out the possibility that an organism without either *mxaF*, *pmoA*, or *mmoX* was responsible for the high-affinity CH_4 oxidation. Unidentified species were present in the enrichment, as shown by a DGGE performed with a universal 16S rDNA primer system (data not shown). In this gel, several bands were visible which did not have the same migration patterns as any of the five isolated organisms described above.

Isolate LR1 was therefore grown in a coculture with the *Variovorax* sp. isolated from the enrichment. The *Variovorax* was included because it stimulated the growth of LR1, although alone it could not consume CH_4 (data not shown). This coculture was grown on <275 ppmv of CH_4 for 4 to 5 months,

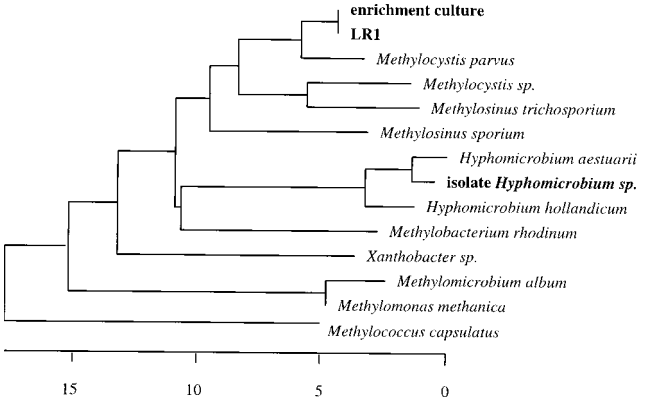

FIG. 6. Unrooted phylogenetic tree, based on derived amino acid sequences of *mxaF* gene fragments, showing the identity of the *mxaF* product from isolate LR1 and that from the high-affinity enrichment culture (grown on <275 ppmv of CH_4) and their relationship to other methylotrophic species. The distance bar represents percent dissimilarity.

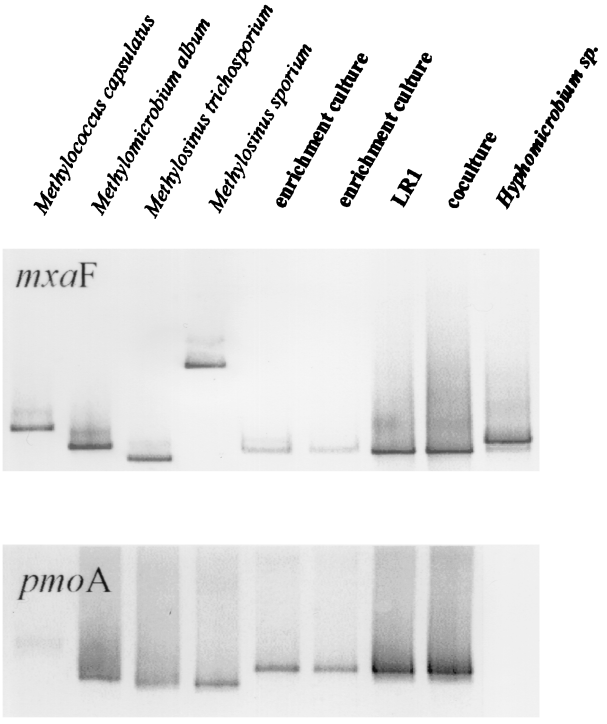

FIG. 7. DGGE patterns of DNA, amplified with primers for *mxaF* and *pmoA*, from selected methanotrophic strains (first four lanes), the high-affinity enrichment culture (grown on <275 ppmv of CH_4), the methanotrophic isolate LR1, a coculture of LR1 with a *Variovorax* sp., and a *Hyphomicrobium* sp. isolated from the enrichment culture.

and kinetic curves were measured as previously described. A $K_{m(app)}$ value of 129 to 188 nM was estimated in this coculture (Table 1). This range overlaps that of the more complex enrichment culture and is considerably lower than any range previously reported for methanotrophic bacteria. Isolate LR1 was therefore probably the high-affinity CH_4 oxidizer in the initial, low-CH_4 enrichment culture (<275 ppmv of CH_4).

DISCUSSION

Methanotrophs in soils exposed to only atmospheric CH_4 possess a higher affinity for CH_4 than do pure cultures of methanotrophic bacteria, which are routinely grown on CH_4 at mixing ratios of 10 to 20% (Table 1). There is no conclusive evidence indicating whether this high-affinity activity is carried out by novel organisms or simply by known methanotrophs behaving in an unknown manner. Recently it was shown that the $K_{m(app)}$ (CH_4) in *M. albus* varied depending on whether the organisms were grown on CH_4 or methanol as an energy source (4). This demonstrates that the $K_{m(app)}$ in known methanotrophic bacteria changes with culture conditions, although neither reported value was near the low-nanomolar $K_{m(app)}$ values measured in upland soil (both were >1 μM) (4). The $K_{m(app)}$ of soils enriched with CH_4 at high mixing ratios has also been shown to vary with the CH_4 supply, but again only in the $K_{m(app)}$ range of >1 μM (19).

Our data suggest two major points: that a type II methanotrophic species in mixed culture can exhibit $K_{m(app)}$ values which vary depending on the CH_4 supply and that the $K_{m(app)}$ of such a culture can approach that measured in upland soils when low CH_4 mixing ratios (<275 ppmv) are used for growth. We produced a methanotrophic enrichment culture which, based on its *pmoA*, *mmoX*, and *mxaF* products, contained a single detectable methanotroph. This organism (LR1) was a type II species of the *Methylosinus*/*Methylocystis* group. In the enrichment culture, and in a coculture containing only LR1 and a *Variovorax* sp., growth on <275 ppmv of CH_4 resulted in $K_{m(app)}$ values of 56 to 188 nM. However, when the CH_4 mixing ratio was raised to 1%, the $K_{m(app)}$ shifted upward to a value typical for methanotrophic isolates (>1 μM [Table 1]).

The organic soil from which our enrichment cultures were made consumes atmospheric CH_4 over much of the year (9). We previously measured $K_{m(app)}$ values of 60 to 280 nM CH_4 in this soil, estimating the value in the absence of inhibitory NH_4^+ as 80 to 90 nM (8). Because the $K_{m(app)}$ values in our cultures grown on <275 ppmv of CH_4 were in the same range as these values, species such as LR1 could be the active methane oxidizers in this soil. It is therefore unnecessary here to postulate novel organisms as the agents of atmospheric-methane oxidation. The 16S rRNA gene sequence, as well as sequences for the functional genes *pmoA* and *mxaF*, which are also useful phylogenetic markers for methanotrophic bacteria (26, 27), all showed that LR1 clusters with other type II species and does not belong to a novel lineage of methanotrophs.

The $K_{m(app)}$ values in our cultures grown on <275 ppmv of CH_4 were lower than the lowest previously reported value for methanotrophic isolates. However, the values in our cultures, and in the organic soil itself, are higher than those measured in many other upland soils. These can be as low as 10 nM (1, 3, 13). This organic soil is periodically methanogenic (9) and possibly supports a methanotrophic flora different from that of other soils. Although organisms such as LR1 may account for atmospheric-CH_4 oxidation in this organic soil, other species may dominate in other soils. On the other hand, the fact that the $K_{m(app)}$ in our cultures was variable suggests that even lower values may be inducible under more oligotrophic conditions.

The CH_4 concentration was a key factor in determining $K_{m(app)}$, but this could be mediated through the MMO enzyme, the methanotroph, or the bacterial community as a whole. Only apparent kinetic coefficients are measured in a study with a mixed microbial community, and it cannot be assumed that these represent true enzyme properties. A variety of reasons could therefore account for the variable $K_{m(app)}$. We propose three possibilities. (i) A modified form of MMO is responsible for high-affinity activity. (ii) The measured $K_{m(app)}$ is diffusion limited and affected by physiological properties of methanotrophs which change with growth conditions. Although the specific CH_4 uptake rate in methanotrophs (10^{-15} mol of CH_4 $cell^{-1}$ h^{-1}) (15) is 100 to 1,000 times lower than the maximum rate of CH_4 diffusion through the cell boundary layer (calculated with the van Smoluchowski equation [21]), diffusion limitation could occur if cells aggregate or if the cell envelope presents a diffusion barrier (21). In a diffusion-limited system the measured $K_{m(app)}$ values are affected by the aggregation, the specific activity, or the envelope structure of the cells. (iii) It is difficult to apply simple Michaelis-Menton kinetics to a complex terreactant enzyme such as MMO. The $K_{m(app)}$ for a substrate is a function of both an equilibrium constant (binding of substrate to enzyme) and a reaction rate constant (decomposition to product). These may be altered by the availability of cosubstrates. For example, it might be expected that the cosubstrate NADH is more limiting in methanotrophic cultures grown on <275 ppmv of CH_4 than in those grown on 1% CH_4. If NADH limitation slowed the decomposition of the MMO-CH_4 complex (although this does not agree with the present model of the sMMO reaction sequence [23]), a lower $K_{m(app)}$ could result (35).

Methane oxidation rates in soil have been stimulated by incubation on >1,000 ppmv of CH_4 (1, 2, 29, 34). Transient or minor increases may follow incubation with less CH_4 (3, 12), but until now no study had produced a long-term methanotrophic enrichment with <300 ppmv of CH_4 (2, 4, 20, 34). It has been suggested that periodic methanogenic events (9) or alternate substrates, such as methanol (4, 17), are necessary for growth and maintenance of the methanotrophs which consume atmospheric CH_4 in soils. The results of the present study demonstrate that methanotrophs are more oligotrophic than previously believed but they do not contradict these theories. Our data suggest that the $K_{m(app)}$ value of known methanotrophic species is dependent on environmental conditions, especially the CH_4 supply, and therefore that the low $K_{m(app)}$ values measured in upland soils do not necessarily imply that novel species of "atmospheric-methane oxidizers" exist.

In summary, we propose that organisms closely related to known type II methanotrophic species contribute to high-affinity atmospheric CH_4 consumption in an organic soil and that the $K_{m(app)}$ value of methanotrophic organisms is determined by the conditions under which they are cultured, especially by the CH_4 supply.

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