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Characterization of Root-Associated Methanotrophs from Three Freshwater Macrophytes: *Pontederia cordata*, *Sparganium eurycarpum*, and *Sagittaria latifolia*†

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Root-associated methanotrophic bacteria were enriched from three common aquatic macrophytes: *Pontederia cordata*, *Sparganium eurycarpum*, and *Sagittaria latifolia*. At least seven distinct taxa belonging to groups I and II were identified and presumptively assigned to the genera *Methylosinus*, *Methylocystis*, *Methylomonas*, and *Methylococcus*. Four of these strains appeared to be novel on the basis of partial 16S ribosomal DNA sequence analysis. The root-methanotroph association did not appear to be highly specific, since multiple methanotrophs were isolated from each of the three plant species. Group II methanotrophs were isolated most frequently; though less common, group I isolates accounted for three of the seven distinct methanotrophs. Apparent K_m values for methane uptake by representative cultures ranged from 3 to >17 μM; for five of the eight cultures examined, apparent K_m values agreed well with apparent K_m estimates for plant roots, suggesting that these strains may be representative of those active in situ.

Söhngen (33) isolated and characterized the first methane-oxidizing bacterium by using enrichments from the leaves of submerged aquatic macrophytes. Subsequently, methanotrophs have been isolated from soils, sediments, and the water column of freshwater and marine systems (18, 20, 24, 35, 36). Methanotrophs and methanotrophic activity have also been described for mytilid mussels harboring bacterial endosymbionts (10, 11, 13). In addition, both in vitro and in situ methane oxidation rates have been documented for various aquatic plant roots (16, 25, 26, 31).

Although root-associated methanotrophy limits methane emission from wetlands to the atmosphere and thus plays an important role in the global methane budget, little is known about the bacteria responsible for this activity. To date, there are no published studies of methanotrophic enrichments or isolates specifically derived from the roots or rhizospheres of aquatic plants. As a result, the similarity of root methanotrophs to isolates from other systems remains unclear; likewise, the host plant specificity of root methanotrophs is unknown. King (25) and Hanson and Hanson (18) report that group II methanotrophs dominate root populations on the basis of signature deoxyribonucleotide hybridization patterns. However, these studies provide no information on the diversity or characteristics of root methanotrophs, nor do they address the extent to which such organisms can be routinely isolated.

We describe here characteristics of root-associated methanotrophs and their distribution among three common aquatic plant species. The populations of root-associated methanotrophs include at least seven distinct taxa, three and four each from the phylogenetically coherent groups I and II, respectively; all of the latter four appear novel, based on partial 16S ribosomal DNA (rDNA) sequence analysis. The isolates most frequently obtained were assigned to group II; group I isolates were rarer, an observation consistent with previous results (25). The various isolates from both groups are similar to extant cultures, based on morphology, colony characteristics on solid media, and physiological attributes. One-half-saturation constants for methane (apparent K_m) are also consistent with previously reported apparent K_m values for sediment-free roots of various freshwater plants and extant cultures (25). Ranges in apparent K_m and V_{max} suggest the possibility that some methanotrophs may be adapted for colonization of the root surface while others may be better adapted for colonization of the root interior.

MATERIALS AND METHODS

Root enrichments. *Pontederia cordata*, *Sagittaria latifolia*, and *Sparganium eurycarpum* were collected from marshes in Bristol and Orono, Maine (9, 26). Methanotrophs on or in approximately 4 g (fresh weight) of sediment-free excised roots were enriched in each of the following nutrient solutions: (i) Higgins nitrate mineral salts medium (NMS) (10.0 mM KNO_3, 6.1 mM Na_2HPO_4, 3.9 mM KH_2PO_4, 0.8 mM Na_2SO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$), (ii) NMS without added copper (NMS [−Cu]), (iii) Higgins ammonium mineral salts (AMS) (same composition as NMS but with 10.0 mM NH_4Cl and no nitrate, and (iv) mineral salts medium with no added nitrogen (−N).

For each of the four media, trace elements were added to give the following final concentrations: 2 μM $ZnCl_2$, 2 μM $CuCl_2$, 1 μM NaBr, 0.5 μM Na_2MoO_2, 2 μM $MnCl_2$, 1 μM KI, 2 μM H_3BO_3, 1 μM $CoCl_2$, and 1 μM $NiCl_2$. Iron was added to autoclaved media as $FeSO_4$ in 1 M HCl to produce a final concentration of 50 μM and a pH between 6.8 and 7.0. NMS (−Cu) and nitrogen-free basic mineral salts media were used to select for group II methanotrophs that fix nitrogen and express soluble methane monooxygenase (sMMO) under copper-limited conditions.

Roots were agitated at 100 rpm in 300 ml of medium in 1-liter flasks with a 30 to 70% methane-air headspace at 32°C. Roots from each plant were incubated in triplicate in each of the four media; this procedure was conducted twice for each plant species, once in September 1994 and once in July 1995.

Culture isolation. Root enrichments were incubated until the medium was turbid. One milliliter from each of the enrichments was transferred to 10 ml of nutrient medium (each of the four described above) in 160-ml culture bottles (incubated at 32°C, with shaking at 100 rpm and a 30 to 70% methane-air headspace). The enrichments were subcultured weekly for approximately 6 months until there were ≤5 distinct morphotypes per culture as determined from phase-contrast microscopy. Subsequent efforts to isolate pure cultures included a series of serial dilutions in liquid media and on mineral salts agar plates with washed Bacto agar (Difco, Inc.). The plates were incubated at 32°C in sealed jars with a 30 to 40% methane headspace. Colonies from the plates were subcultured every 2 to 3 weeks and transferred to liquid and solid media. Cultures containing ≤3 discrete morphotypes as determined by microscopic examination were used for further characterization.

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Culture characterization. BacLight viability and Gram stains (Molecular Probes, Inc.) were used for morphological and Gram reaction analysis. One milliliter of exponentially growing broth culture was transferred to 1.5-ml microcentrifuge tubes. BacLight stain was added to the tubes, which were then incubated in darkness for 20 min according to the manufacturer's instructions; subsamples of the stained cultures were transferred to agar-coated slides and examined with a Zeiss Axioscope with epifluorescence illumination and 400× Achrostigmat and 1,000× Plan-neofluar phase-contrast objectives. A small volume of culture was heat fixed on slides for poly-β-hydroxybutyrate staining with 0.03% (wt/vol) Sudan black B and a 0.5% safranin counterstain (15). Loeffler methylene blue was used for staining polyphosphate inclusions. Broth cultures for these stains were 2 to 3 weeks old. A Difco Gram stain kit was also used on fresh cultures. Bacterial cysts were stained with neutral red and light green S.F. yellowish dyes (15) with broth cultures at least 3 weeks old. Capsules were stained with India ink according to the Duguid method (15).

Exospore formation was determined with 2-week-old broth cultures grown in 10 ml of Higgins NMS at 32°C in 160-ml culture bottles with a 30% methane headspace. A volume of culture (0.5 ml) was transferred to fresh medium to produce a set of controls; a second set was pasteurized at 80°C for 20 min. Initial cell density was determined by measuring absorbance at 600 nm on a Beckman DU 640 spectrophotometer; absorbance was assayed periodically for an additional 3 weeks. Exospore formation was also determined by microscopy.

Lysis in 0.2 and 2% sodium dodecyl sulfate (SDS) was determined with 1 ml of unwashed culture. Microcuvettes containing culture and SDS were vortexed and assayed spectrophotometrically (A_{600}). Colony morphology and pigmentation were determined by examining 1-week-old NMS agar plate cultures. Plates also were incubated in air and compared to plates incubated in methane to ensure positive identification of methanotrophic colonies.

Physiological assays. sMMO production was determined by using a modification of the naphthalene oxidation assay of Brusseau et al. (6). Cell suspensions were diluted to an A_{600} of 0.2. One milliliter of culture was transferred to a 10-ml screw-cap tube, and 1 ml of saturated naphthalene solution (about 234 μM at 25°C) was added. The solution was incubated at 25°C and shaken at 200 rpm for 1 h. After incubation, 100 μl of fresh 0.2% tetrazotized *o*-dianisidine was added. The absorbance of the resulting solution was read at 525 nm. The intensity of diazo dye formation was proportional to the oxidation of naphthalene (6). Cultures were considered positive for sMMO when samples appeared blue.

Temperature and pH response measurements were conducted with fresh, washed cultures. Cells were grown to an A_{600} of 0.2 to 0.4 and harvested in exponential phase by centrifugation for 10 min at 4°C and 9,500 × *g*. The pellets were washed twice in Higgins neutral phosphate buffer (NPB) (6.1 mM Na_2HPO_4, 3.9 mM KH_2PO_4 [pH 7]) and resuspended in NPB. Nine milliliters of Higgins nutrient medium was inoculated with 1 ml of washed culture and incubated with a 30% methane headspace at the desired temperature or pH in 60-ml culture bottles stoppered with green neoprene stoppers. Cultures were agitated at 100 rpm. Absorbances were assayed periodically and compared to the initial readings. For the pH assays, pH was adjusted after the addition of Fe, trace metals, and salts. For pHs of ≤7.0, a citrate-phosphate buffer (5 mM citric acid, 10 mM Na_2HPO_4) was used; for pHs of ≥7.0, a phosphate buffer solution (10 mM Na_2HPO_4, 10 mM NaH_2PO_4) was used.

DNA analysis. Selected cultures (isolates 2, 7, 8, 12, 13, 19, 20, 22, and 23 and *Methylomonas albus* BG8 and *Methylosinus trichosporium* OB3b) were grown in Higgins medium with nitrate and a 20% methane headspace. The cultures were harvested by centrifugation and washed twice with 10 mM phosphate buffer (pH 7). The final pellets were resuspended in 1× Tris-EDTA buffer (TE) and stored frozen (−20°C) prior to further analysis. A subsample of thawed cell suspension was subjected to three cycles of freezing (−70°C) and thawing (65°C) and then incubated with lysis buffer. DNA from cell lysates was extracted with phenol-chloroform-isoamyl alcohol and purified by standard methods (2). DNA was stored frozen (−20°C) in TE prior to PCR assays.

PCR of methanotroph DNA was based on the use of signature oligonucleotides as specific primers. The specificity of the two primers has been described previously by Brusseau et al. (7); briefly, 1034-SER (5′-CCA-TAC-CGG-ACA-TGT-CAA-AAG-C-3′) hybridizes with the group II methanotrophs, and 1035-RuMP (5′-GAT-TCT-CTG-GAT-GTC-AAG-GG-3′) hybridizes with group I methanotrophs other than those in the genus *Methylococcus*. These oligonucleotides were used as reverse primers in combination with the forward primer, 530f (5′-GTG-CCA-GCM-GCC-GCG-G-3′), which hybridizes with eubacteria in general (34). A typical PCR was based on a 100-μl volume with 1 to 2 U of *Taq* polymerase (Promega, Inc.), 15 mM $MgCl_2$, 100 ng of template, and approximately 100 μM of one of the pairs of forward-reverse primers. Amplification conditions included the following (after a hot start): 5 min at 95°C (1 cycle); 94°C denaturation, 55°C annealing, 72°C polymerization (30 cycles); and 72°C final extension (10 min). PCR products were electrophoresed in 2% NuSieve 3:1 agarose (FMC Corporation) with 1× tris-borate-EDTA buffer (TBE) and visualized with UV illumination after staining with ethidium bromide (27a). All products were of the expected size (about 500 bp) as determined by comparison with the electrophoresis of a set of PCR markers (Bio-Rad Laboratories, Inc.). Deionized water controls were always negative, as were samples of *M. albus* BG8 and *M. trichosporium* OB3b DNA when amplified with the group II and group I primers, respectively.

PCR products were picked from the agarose gels and extracted after treatment with agarase according to the manufacturer's (FMC Corp.) instructions. A subsample of the amplified DNA was sequenced at the University of Maine Sequencing Laboratory with an ABI 373A sequencer and a Perkin-Elmer ABI prism dye terminator cycle sequencing kit with Amplitaq DNA polymerase. The resulting sequences, along with various sequences from GenBank, were aligned with CLUSTAL V; alignments were also compared with those previously used by Brusseau et al. (7). Phylogenetic relationships were determined from 432 bp of sequence (corresponding to *Escherichia coli* positions 732 to 1194) with programs from the PHYLIP version 3.5 package (DNAML, DNAPARS, DNADIST, SEQBOOT, and CONSENSE [12]).

Kinetic analyses. Kinetic assays were conducted with *M. trichosporium* OB3b and root isolates dominated by a single morphotype (2, 4, 8, 10, 12, 13, 19, 20). Cultures were grown at 32°C in 1-liter flasks with 200 ml of Higgins NMS and were shaken at 150 rpm. Cells were grown to an A_{600} of 0.2 to 0.4 and harvested by centrifugation for 10 min at 10°C and 9,500 × *g*. The pellets were washed with Higgins NPB (10 mM, pH 7) and recentrifuged. The final pellets were resuspended in 20 ml of 10% NMS (A_{600}, 0.02 to 0.04), and a volume was transferred to 160-ml cultures bottles to yield final bacterial concentrations in four ranges of approximately 0.02 to 0.04, 0.04 to 0.06, 0.08 to 0.1, and 0.1 to 0.15 mg ml^{-1} in a total volume of 5 ml. These bacterial concentrations were used for incubations with dissolved methane concentrations of 0.15 to 1, 4 to 8, 8 to 16, and >32 μM, respectively. The cultures were incubated horizontally in triplicate with vigorous shaking at 32°C. Headspace samples of 0.2 cm^3 were collected with 1-ml disposable syringes and needles for methane analysis with a Shimadzu 14A gas chromatograph and a flame ionization detector operated at 150°C. Methane was separated with a Porapak Q column in series with a wide-bore capillary column (DB-1; 30 m by 0.53 mm [outside diameter]) (J&W Scientific, Inc.). Samples were collected at 10- to 20-min intervals during a 2- to 4-h incubation. Kinetic parameters (V_{max} and apparent K_m) were determined by fitting data to the Michaelis-Menten model by using the nonlinear curve-fitting algorithm of Kaleidagraph version 3.0.3 (Adelbeck Software). Cell densities were measured at the beginning and termination of the experiment to determine if the cultures had grown significantly during the sampling period.

RESULTS

Root enrichments. Enrichments for methanotrophs were successful in each of 24 attempts, from which 13 cultures were selected for characterization. The remaining 11 cultures were morphologically indistinguishable from the vibrioid exospore formers described below and were not further characterized. Although pure cultures were not obtained from any of the enrichments, the majority existed in stable consortia, with only one consort present at very low densities. In some cases, the consorts were not evident by microscopy but grew on various solid media incubated without methane. The consorts used diverse organic compounds as carbon and energy sources, including organic acids, amino acids, sugars, and methanol. Because of its distinctive morphology, it was evident that a *Hyphomicrobium* sp. occurred as a consort in some of the cultures.

Methanotroph characterization. All methanotrophs were gram negative and mesophilic, growing best at temperatures of >20°C and at pH values between 6 and 7 (Table 1). Six cultures were dominated by encapsulated rosette-forming vibrios (strains 7, 10, 13, 20, 23, and 24) 2 to 3 μm long and 1 to 1.5 μm in width; two of the 6 (strains 13 and 20) were motile. During stationary phase, all of the rosette-forming vibrios elongated to a comma shape and produced encapsulated exospores relatively quickly (after 4 to 5 days) in liquid cultures. Four of the six rosette-forming vibrio cultures grew after pasteurization at 80°C for 20 min. Colonies of the rosette-forming vibrios on NMS agar plates were low convex with entire edges, of butyrous consistency, and buff colored. All of the rosette-forming vibrios produced sMMO (based on naphthalene oxidation) in a copper-limited medium (Table 1).

Strains 2, 4, 8, and 12 were rods (0.6 to 1.25 μm by 2.0 to 3.0 μm) that did not produce sMMO in copper-limited medium. Strain 8 was distinguished from the others by the absence of cysts, poly-β-hydroxybutyrate, and polyphosphate inclusions. None of these strains formed rosettes. Capsules were present for strains 4, 8, and 12. Most colonies on NMS agar were

TABLE 1. Phenotypic characteristics of methanotrophic isolates from the roots of *P. cordata*, *S. eurycarpum*, and *S. latifolia*

Parameter	Characteristic for isolate:												
	2	4	8	12	7	10	13	20	23	24	21	22	19
Cell morphology													
Cocci, paired													+
Bacilli	+	+	+	+							+	+	
Vibrioid					+	+	+	+	+	+			
Length <1–<2.0 μm											+	+	+
Length 2.0–3.0 μm	+	+	+	+	+	+	+	+	+	+			
Width 0.6–1.25 μm	+	+	+	+	+	+	+	+	+	+	+	+	+
Poly-β-hydroxybutyrate	−	+	−	−	−	−	−	−	−	−	−	+	+
Polyphosphate	+	−	−	+	−	−	−	−	−	−	+	−	+
Cyst formation	+	+	−	+	−	−	−	−	−	−	+	+	+
Exospore formation	−	+	+	+	+	+	+	+	+	+	−	−	−
Group, presumptive	II	II	II	II	II	II	II	II	II	II	I	I	I
Gram stain	−	−	−	−	−	−	−	−	−	−	−	−	−
Capsule	−	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	−	−	−	−	−	+	+	−	−	+	+	−
Rosette formation	−	−	−	−	+	+	+	+	+	+	+	−	+
Chain formation	+	−	−	−	−	−	−	−	−	−	+	−	+
Colony morphology													
Translucent													
Opaque	+	+	+	+	+	+	+	+	+	+	+	+	+
Low convex		+	+	+	+	+	+	+	+	+	+	+	+
High convex	+												
Lobate or irregular edges	+												
Entire edges		+	+	+	+	+	+	+	+	+	+	+	+
Butyrous consistency		+	+	+	+	+	+	+	+	+	+	+	+
Mucoid consistency	+												
Pigmentation													
White or buff	+	+	+	+	+	+	+	+	+	+			+
Pink or rose											+		
Salmon to orange												+	
Growth in static culture as evenly dispersed clumps or pellicles	+	+	+	+	+	+	+	+	+	+	+	+	+
Physiological characteristics													
sMMO	−	−	−	−	+	+	+	+	+	+	−	−	−
Lysis by 0.2% SDS	−	−	−	−	−	−	−	−	−	−	+	+	−
Lysis by 2% SDS	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 10°C	+	−	−	+	ND	−	−	+	+	−	+	−	−
Growth at 20°C	+	+	+	+	ND	+	+	+	+	+	+	+	+
Growth at 30°C	+	+	+	+	ND	+	+	+	+	+	+	+	+
Strain no.	2	4	8	12	7	10	13	20	23	24	21	22	19

[a] ND, no data.

opaque, low convex, of butyrous consistency, and buff colored. However, strain 2 differed by forming a high-convex, mucoid colony with irregular edges.

Strain 19 consisted of encapsulated, nonmotile paired cocci (0.6 to 1 μm by 0.5 to 1.0 μm) which often formed tetrads. Cysts were formed in older cultures. Colonies were buff colored and low convex with entire edges. This strain did not produce sMMO in copper-limited medium.

Strains 21 and 22 were encapsulated, small, motile rods that formed *Azotobacter*-like cysts and were sMMO negative in copper-limited medium. Cells lysed in 0.2% SDS. Strain 21 formed bright pink, low-convex colonies with entire edges. Strain 22 formed salmon-to-orange colonies of the same general description. Both strains formed pellicles in static and agitated cultures.

Strains 2, 4, 7, 8, 10, 12, 13, 20, 23, and 24 yielded a PCR product with the 1034-SER but not the 1035-RuMP primer; on this basis, these strains are assigned to the group II methanotrophs. Strain 22 yielded a product only with the 1035-RuMP primer and is therefore assigned to the group I methanotrophs. No amplification product was obtained from strain 19, but its similarity to the genus *Methylococcus* indicates placement in group I. Strain 21 was not assayed by PCR, but its similarity to the genus *Methylomonas* also indicates that it is probably in group I. The remaining strains (1, 3, 5, 6, 9, 11, and 14 to 18) were indistinguishable morphologically from the vibrioid exospore formers (e.g., strains 7, 10, 13, 20, 23, and 24) and are presumably group II methanotrophs.

Comparison of partial 16S rDNA sequences from selected strains indicated that many were equivalent (e.g., strain 7 was equivalent to strain 10; strain 13 was equivalent to strains 20, 23, and 24; and strain 8 was equivalent to strain 12). Maximum-likelihood phylogenetic analysis based on a 100-iteration bootstrap data set of aligned sequences for the root strains and other methanotrophs (Fig. 1) indicated that strains 2 and 12 were distinct but related to previously reported group II sequences from a peat bog and that strains 10 and 13 were

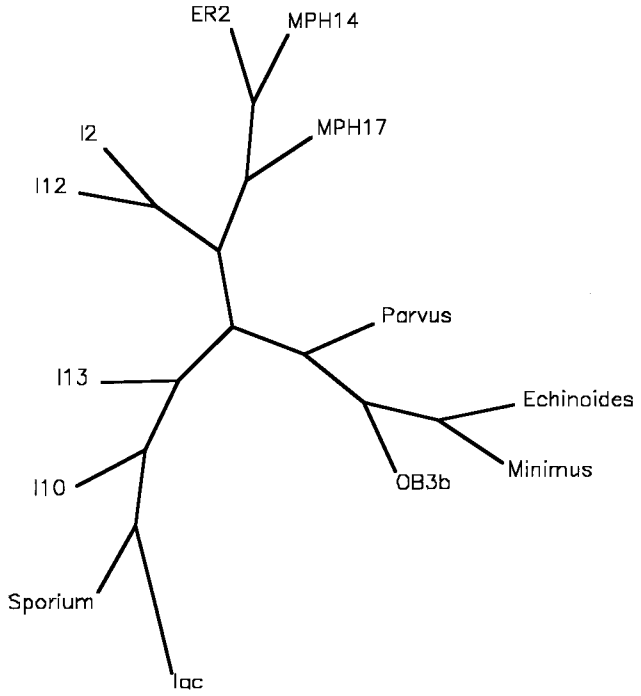

FIG. 1. Unrooted consensus tree derived from a maximum-likelihood analysis of a 100-iteration bootstrap data set of partial 16S rDNA sequences for selected group II methanotrophs and root isolates; analysis was performed with the SEQBOOT, DNAML, and CONSENSE packages of PHYLIP version 3.5. MPH14 and MPH17 represent sequences obtained from group-specific PCR of a genomic extract from bog peats (28). Strains I2 and I12 are presumed *Methylocystis* spp., and I10 and I13 are assigned to the genus *Methylosinus*. Parvus, *Methylocystis parvus*; Echinoides, *Methylocystis echinoides*; Minimus, *Methylocystis minimus*; Sporium, *Methylosinus sporium*; lac, *Methylosinus* sp. strain lac; ER, *Methylosinus* sp. strain ER2.

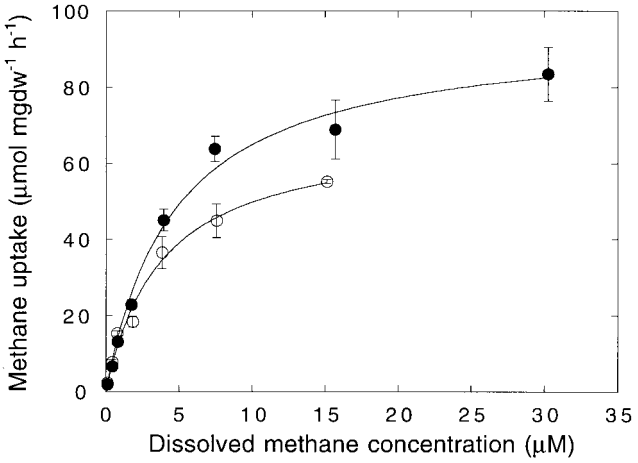

FIG. 2. Methane uptake rate versus dissolved methane concentration for two presumed *Methylocystis* spp. (strain 4 [●] and strain 8 [○]); values represent means ± 1 standard error. Plots are representative of the seven additional strains assayed.

related but distinct from other root methanotrophs and group II isolates.

Of the seven distinct strains identified from the various characterizations, four were obtained from *P. cordata* (Table 2), including two vibrios (strains 12 and 13), a paired coccus (strain 19), and a rod (strain 22). Three strains each were obtained from *S. eurycarpum* (a vibrio [strain 24] and two distinct rods [strains 2 and 21]) and *S. latifolia* (two vibrios [strains 10 and 20] and one rod [strain 4]). Note that some of the strain numbers on different plant taxa represented equivalent methanotrophs (e.g., strains 13 and 20). Both group I and group II methanotrophs were obtained from *P. cordata* and *S. eurycarpum*, while only group II isolates were cultured from *S. latifolia*. Group I and group II methanotrophs were also obtained from copper-sufficient NMS, while only group II isolates were enriched from copper-limited NMS. AMS and nitrogen-free media supported one group I methanotroph, with group II strains otherwise dominant.

Kinetic analyses. The methanotrophs used for the kinetic analyses did not grow measurably during the assays. Methane oxidation kinetics conformed to a Michaelis-Menten model (Fig. 2). V_{max} values ranged from 42.2 to 118 μmol of methane mg (dry weight)$^{-1}$ h^{-1}; apparent K_m values ranged from 3.0 to 17.0 μM. The V_{max} and apparent K_m for *M. trichosporium* OB3b were 24 ± 1.5 μmol mg (dry weight)$^{-1}$ h^{-1} and 1.0 ± 0.3 μM, respectively. Strains 2, 8, 13, and 20 had similar V_{max} and apparent K_m values, while cultures 10, 12, and 19 had the highest apparent K_ms (Table 3). V_{max} values and apparent K_ms were significantly correlated when they were plotted as pairs for all of the cultures ($r = 0.72$; $P = 0.03$) (Fig. 3).

DISCUSSION

In spite of the global importance of aquatic plants for the production, transport, and oxidation of methane, little is known about the specific groups of root-associated bacteria that directly affect methane dynamics. The results presented here provide new insights into the taxonomic diversity of root-associated methanotrophs and some of the important characteristics of these organisms (e.g., methane uptake kinetics). Although the relative importance in situ of the various methanotrophs obtained during this study is as yet unknown, the

TABLE 2. Distribution of presumed methanotrophic strains from the roots of three aquatic macrophytes under four enrichment regimes

Enrichment	Distribution of strains[a] from:		
	P. cordata	*S. eurycarpum*	*S. latifolia*
NMS	*Methylococcus* sp. (19), *Methylocystis* sp. (12)	*Methylocystis* sp. (8), *Methylomonas* sp. (21)	*Methylocystis* sp. (4), *Methylosinus* sp. (20)
NMS, Cu−	*Methylosinus* sp. (6, 13)	*Methylosinus* sp. (5, 15)	*Methylosinus* sp. (1, 14)
AMS	*Methylosinus* sp. (16)	*Methylocystis* sp. (2), *Methylosinus* sp. (18)	*Methylosinus* sp. (7, 17)
−N	*Methylosinus* sp. (9), *Methylomonas* sp. (22)	*Methylosinus* sp. (11, 24)	*Methylosinus* sp. (10, 23)

[a] Genus designations are presumptive and based on strain descriptions and assignments in the text; strain numbers are given in parentheses.

TABLE 3. Kinetic parameters for methane oxidation by selected methanotrophic strains from sediment-free roots of *S. eurycarpum*, *P. cordata*, and *S. latifolia* and by *Methylosinus trichosporium* OB3b

Strain	V_{max}[a] (mean ± 1 SE)	$K_{m\ app}$ (μM) (mean ± 1 SE)	$V_{max}\ K_{m\ app}^{-1}$
2	55.0 ± 7.0	7.2 ± 2.3	7.6
4	102.0 ± 5.0	4.3 ± 0.7	23.7
8	66.2 ± 8.0	3.0 ± 1.0	22.1
12	118.0 ± 11.0	13.2 ± 3.8	8.9
19	60.0 ± 8.0	13.0 ± 3.7	4.6
10	133.0 ± 25.0	17.0 ± 10.0	7.8
13	42.2 ± 6.4	3.4 ± 1.6	12.4
20	71.2 ± 3.0	6.8 ± 1.0	10.5
OB3b	24.0 ± 1.5	1.0 ± 0.3	24.5

[a] Expressed in micromoles per milligram (dry weight) per hour.

dominance of group II forms in the enrichments is consistent with earlier studies that showed a greater abundance of group II than of group I based on signature oligonucleotide hybridization to 16S rRNA in genomic root extracts.

Most of the methanotrophs obtained in this study (e.g., strains 7, 10, 13, 20, 23, and 24) have been assigned to the genus *Methylosinus* on the basis of various morphological and physiological characteristics. Characteristics shared by these strains and the genus *Methylosinus* include Gram reaction (negative); lack of motility; vibrioid morphology; size (2 to 3 μm by 1.0 to 1.5 μm); formation of rosettes and exospores; expression of sMMO in copper-limited media; absence of polyphosphate, poly-β-hydroxybutyrate, and cysts; and colony morphology and color (low convex and buff colored).

Although the morphology of the vegetative cells is most similar to descriptions of *Methylosinus sporium* (5, 14, 19, 36), the exospores of *M. sporium* lack capsules (36), in contrast to consistent encapsulation for the isolates described here. Analysis of partial 16S rDNA sequences also supports the assignment of these strains to the genus *Methylosinus*, as indicated by their relationship to *M. sporium* and *Methylosinus* strain LAC. However, the two distinct but closely related root methanotrophs represented by strains 10 and 13 clearly differed from other *Methylosinus* strains, including two apparently novel sequences recently reported from a peat bog (Fig. 1) (28).

A second taxonomic grouping encompasses strains 2, 4, 8, and 12. Size, morphology, lack of motility, and response to 0.2% SDS suggest an affinity to the genus *Methylocystis* (5). The lack of sMMO expression is consistent with *Methylocystis parvus*. In addition, polyphosphate, poly-β-hydroxybutyrate, motility, and capsule formation are variable in the genus *Methylocystis* (5) as they are in strains 2, 4, 8, and 12. Results of a phylogenetic analysis indicate that strains 2, 4, 8, and 12 (strain 4 was not sequenced but is otherwise identical to 8 and 12) form a distinct group more closely related to sequences from a peat bog in the United Kingdom (MPH14 and MPH17) than to the *Methylosinus*-like strains from roots or other known methanotrophs (Fig. 1). In addition, strain 2 differs from strains 8 and 12, which is consistent with its morphological and physiological characteristics (e.g., lack of spores and capsules [Table 1]). Thus, this second grouping of strains differs from the first and likely includes at least two distinct taxa as well (e.g., strain 2 versus strains 4, 8, and 12).

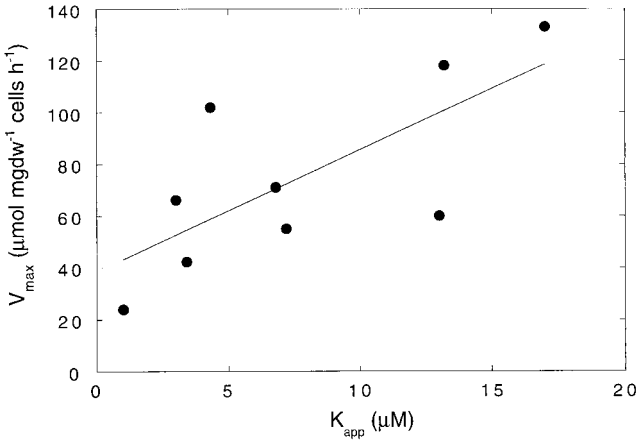

FIG. 3. V_{max} versus $K_{m\ app}$ for selected methanotrophic strains ($r = 0.72$; $P = 0.03$); values are summarized in Table 3.

A third grouping is suggested by the characteristics of strains 21 and 22. Morphology, size, encapsulation, motility, pigmentation, and *Azotobacter*-like cysts strongly suggest affinities with the genus *Methylomonas*. Pigmentation and polyphosphate inclusions in strain 21 and poly-β-hydroxybutyrate inclusions in strain 22 indicate their assignment to *Methylomonas methanica* and the closely related *Methylomonas aurantiaca* and *Methylomonas fodinarum*, respectively (4). The latter assignment is supported by 16S rDNA sequence analyses which show a high degree of identity between strain 22 and *M. aurantiaca* and *M. fodinarum.*

Strain 19 is most similar to the genus *Methylococcus*. Encapsulation, lack of motility, the presence of paired cocci (0.6 to 1 μm by 0.5 to 1.0 μm) that often formed tetrads, and the presence of polyphosphate and poly-β-hydroxybutyrate are all consistent with the characteristics of this genus (5). Colony characteristics and the lack of lysis in 0.2% SDS for strain 19 are also similar to characteristics of this genus. However, in contrast to the well-known production of sMMO by *Methylococcus capsulatus* Bath, strain 19 is sMMO negative. The inability of the 1035-RuMP primer to amplify DNA from strain 19 is consistent with previous reports of the response of *Methylococcus* spp. (7) to this primer and supports the generic assignment, albeit indirectly.

Distribution and diversity of root-associated methanotrophs. Although at least seven distinct taxa were enriched from three plant species in this study, the true level of methanotroph diversity is likely higher, since enrichments generally select against some strains. Several of the methanotrophs obtained from the enrichments were cosmopolitan, appearing in all nutrient regimens for all plants. These may be representative of the most common taxa, or at least the taxa that are generally distributed and most competitive in enrichments. In contrast, several strains appeared more restricted in their distribution, occurring only in a specific medium or on a single plant species. These taxa may be representative of the least-abundant methanotrophs.

The cosmopolitan distribution of several of the taxa suggests that at least some root-methanotroph associations may be opportunistic. This differs from other microbe-wetland root associations that involve much more specific host interactions (e.g., associations based on nitrogen-fixing actinomycetes [32]). Whether the few, more specific methanotroph associations known for animals (10, 11) represent exceptions among a larger number of opportunistic associations is unclear.

Several lines of evidence, including results from PCR assays with group I- and group II-specific primers, indicate that most of the root methanotrophs belong to group II (four of the seven distinct taxa and 21 of 24 total isolates). Group I and

group II methanotrophs occur on *P. cordata* and *S. eurycarpum* roots, but only group II was isolated from *S. latifolia*. However, this probably does not accurately reflect methanotroph diversity in situ, since PCR of genomic DNA extracts from *S. latifolia* reveals both groups (37).

The availability of methane, oxygen, nitrogen, and copper probably plays a major role in determining methanotrophic population structure (1). Group II methanotrophs may dominate in environments where growth rates are restricted periodically by deprivation of nutrients, particularly nitrogen (17). Under such conditions, nitrogenase expression presumably provides a selective advantage for group II methanotrophs. Group II methanotrophs might also be expected to dominate in systems with an abundance of methane, such as wetlands, since they grow more efficiently than group I methanotrophs at high substrate concentrations (1, 7, 25, 30).

Kinetic analyses. The V_{max} and apparent K_m reported here (Table 3) for *M. trichosporium* OB3b (24 ± 1.5 μmol of methane mg [dry weight]$^{-1}$ h^{-1} and 1.0 ± 0.3 μM, respectively) agree well with results reported by others, especially the values of Joergensen and Degn (22) that were based on membrane inlet mass spectroscopy, which eliminates phase transfer limitations. Root methanotroph V_{max} values (42 to 133 μmol mg [dry weight]$^{-1}$ h^{-1}) significantly exceed those of other methanotrophs characterized to date (3, 18, 22). Using root methanotroph V_{max} values and the observed maximal methane oxidation rates of washed roots in vitro (1 to 10 μmol g [dry weight]$^{-1}$ h^{-1} [25]), one can estimate the population size necessary to account for root activity. The values thus obtained, 3×10^7 to 9.5×10^8 cells g (dry weight)$^{-1}$ of root, are clearly speculative, but they indicate that methanotrophs likely represent a significant fraction of the root microbiota.

Apparent K_m values (about 3 to 7 μM [Table 3]) for 5 of the 8 strains assayed are comparable to estimates obtained for the washed roots of a variety of wetland macrophytes (3 to 6 μM) (25), which suggests that at least some of the root enrichments may be representative of the dominant methanotrophs in situ. Several strains with somewhat higher values (>10 μM) may represent less important populations. The apparent K_ms of the root strains are also comparable to values obtained for other aquatic systems, including lake and wetland sediments (about 2 to 10 μM) (8, 23, 27, 29) and peats (about 4 μM) (38).

In general, neither V_{max}, apparent K_m, nor V_{max}/apparent ($K_{m\ app}$) K_m varied consistently among the strains (Table 3). For instance, strains 4, 8, and 12 are very similar taxonomically; however, although V_{max} is comparable for 4 and 12, the apparent K_ms for these strains differ. Likewise, the apparent K_ms for strains 4 and 8 are comparable, but the V_{max}s differ. The most consistent trend among the kinetic results is the positive correlation ($r = 0.72$; $P = 0.03$) between V_{max} and $K_{m\ app}$, which suggests that a lower uptake affinity accompanies increased uptake capacity for methane. This relationship may reflect methanotrophic ecological strategies. Strains with a relatively low $K_{m\ app}$ may have a competitive advantage in the root interior, where methane concentrations are low, while strains with a higher $K_{m\ app}$ may have an advantage in the rhizoplane. Future efforts based on strain-specific fluorescent probes could prove useful in determining the relationship between kinetic characteristics and methanotroph microzonation.

In summary, representatives of four methanotrophic genera and both of the phylogenetically coherent groups (I and II) have been enriched from the roots of aquatic macrophytes. Group II taxa (e.g., *Methylocystis* and *Methylosinus* spp.) were most abundant and included four potentially novel strains, based on partial 16S rDNA analysis. The isolation and characterization of root methanotrophs provide a basis for understanding the physiological limitations of methane oxidation in situ, as well as material for the development of specific nucleic acid and immunological probes for assessing in situ distribution and abundance.

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