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EXPERIMENTAL ARTICLES

Methanotrophs and Methylobacteria Are Found in Woody Plant Tissues within the Winter Period

N. V. Doronina, E. G. Ivanova, N. E. Suzina, and Yu. A. Trotsenko[1]

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia

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Abstract—Samples of tree seeds, buds, and needles collected within the winter period at ambient temperatures from –11 to –17°C were analyzed for the presence of methylotrophic microflora. Thin sections of blue spruce needles were found to contain bacteria morphologically close to pink-pigmented methylobacteria. The methylobacteria that were isolated in pure cultures from samples of linden seeds and buds and pine and blue spruce needles, as well as of lilac, maple, and apple buds, were classified into the genera *Methylobacterium* and *Paracoccus* based on the data of morphological studies, enzyme assay, and DNA–DNA hybridization analysis. The methanotrophs that were isolated in pure cultures from samples of linden buds and blue spruce needles were referred to the genus *Methylocystis* based on the data of morphological studies, enzyme assay, DNA–DNA hybridization, and the phylogenetic analysis of the particulate methane monooxygenase gene *pmoA* sequences. The inference is made that aerobic methylotrophic bacteria are permanently associated with plants. At the beginning of the vegetative period in spring, the phyllosphere of coniferous and deciduous trees is colonized by methylotrophic bacteria that have wintered inside plant tissues.

Key words: methylobacteria, methanotrophs, tree seeds, buds, and needles.

Aerobic methylotrophic bacteria, which utilize methane (methanotrophs) or its oxidized and substituted derivatives (methylobacteria), are often encountered in the phyllosphere and rhizosphere of plants [1, 2]. Since methanol, methylamines, methylhalides, and carbon disulfide are common volatile metabolites of plants, methylotrophic bacteria can successfully compete with other plant-associated microorganisms. Moreover, due to their ability to produce cytokinins and auxins, methylotrophic bacteria can be considered phytosymbionts [1, 3]. Experiments showed that methanotrophs and methylobacteria can stimulate the growth and development of gnotobiotic plants cultivated in vitro [4, 5]. The pink-pigmented facultative methylobacteria (PPFM) of the genus *Methylobacterium* efficiently colonize the phyllosphere of many plants over the growing season [1, 6]. Pirttila *et al.* detected bacteria of the genus *Methylobacterium* in the buds of scotch pine (*Pinus sylvestris* L.) by the method of in situ hybridization with 16S rRNA–specific oligonucleotide probes [7]. The nonpigmented methylobacterium *Methylobacterium nodulans,* isolated from the rhizosphere of *Leguminosae* plants, was found to nodulate and fix nitrogen in symbiosis with legumes [2]. In recent studies, we showed that the nonpigmented methylobacteria *Methylovorus mays* [8] and *Paracoccus kondratievae* [9], isolated from the phyllosphere and rhizosphere of corn, are able to synthesize cytokinin and auxin phytohormones [1, 3].

Romanovskaya *et al.* [6] suggested that PPFM colonize the phyllosphere of plants in spring, being carried by airborne soil particles. In contrast, our observations showed that methylotrophic bacteria are permanently associated with plants over the entire year.

This work is an attempt to detect methanotrophs and methylobacteria in woody plant tissues over the winter period.

MATERIALS AND METHODS

The objects of study were samples of the seeds and buds of the linden *Tilia cordata* L.; the buds of the lilac *Syringia glauca* L., the maple *Acer platanoides* L., and the apple *Malus domestica* L.; and the needles of the pine *Pinus sylvestris* L. and the blue spruce *Picea pungens* var. *glauca* L. The samples were collected in February 2002 and 2003 in the territory of Pushchino, Moscow oblast, at temperatures between –11 and –17°C. The microbial DNA obtained was subjected to comparative analysis with that of the type strains of the methylotrophic bacteria *Methylobacterium extorquens* NCIMB 9399^T and *Paracoccus denitrificans* ATCC 17741^T, as well as with *Methylobacter bovis* 98, *Methylomonas methanica* S1, *Methylomicrobium album* BG8, *Methylococcus capsulatus* Bath, *Methylosinus*

[1] Corresponding author; e-mail: trotsenko@ibpm.pushchino.ru

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trichosporium OB3b, *Methylocystis parvus* OBBP, and *Methylocystis echinoides* 2 VKM B-2128.

The isolation of methylotrophic bacteria in enrichment and pure cultures. Plant samples (0.5 g) were placed in 700-ml Erlenmeyer flasks with 200 ml of either K or P mineral medium and incubated at 29°C on a shaker (120 rpm) for 5 days. Methylobacteria were grown in liquid or on solid (agar) K medium [8] with 0.5% (v/v) methanol or 0.3% (w/v) methylamine. Methanotrophs were grown in liquid or on solid (agar) P medium in an atmosphere of CH_4–air (1 : 1) [10]. The enrichment cultures of methanotrophs and methylobacteria were subcultured two times. Pure cultures were obtained by the method of exhausting plating of enrichment cultures onto K or P agar media. The isolated colonies were transferred to agar slants. The culture purity of isolated methylotrophic bacteria was tested by examining the total preparations and sections of colonies and cells with light and electron microscopes. In addition, the purity of methanotrophic cultures was tested by the absence of growth on organic media (nutrient agar and glucose–potato agar).

Phenotypic analysis. To prepare thin sections, plant and bacterial samples were fixed in a solution of glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, washed three times in the same buffer, refixed by incubation in 1% OsO_4 at 4°C for 4 h, and dehydrated in a series of alcohol solutions. The preparation was then embedded in Epon-812 epoxy resin. After curing, it was cut into thin sections, which were mounted onto grids and contrasted with uranyl acetate and lead citrate [11]. Some cell preparations were negatively stained with 0.3% phosphotungstic acid (pH 7.2). The thin sections thus obtained were examined with a JEM-100B electron microscope (JEOL, Japan), which was operated either at 80 kV (thin sections of plant tissues and bacterial cells) or at 60 kV (negatively stained cells).

The indicative and key enzymes of C_1 metabolism were assayed by conventional methods [12].

DNA analysis. DNA was isolated as described earlier [8]. PCR analysis was carried out with the total DNA of the primary methanotrophic enrichment subcultures grown in P medium and the oligonucleotide primers to the particulate methane monooxygenase gene *pmoA* and the soluble methane monooxygenase gene *mmoX* [13, 14]. The composition of the methanotrophic enrichments was determined by PCR with group-specific primers [13, 15, 16]. PCR was carried out in a Hybaid thermal cycler (United Kingdom) with the initial DNA denaturation step at 95°C for 2 min, followed by 25 cycles of DNA denaturation at 94°C for 40 s, primer annealing at 55°C for 40 s, and primer extension at 72°C for 40 s, with the final extension step at 72°C for 4 min. The reaction mixture (30 μl) contained 0.5 μl of DNA preparation and 5 pmol of the respective primers in 10 mM Tris–HCl buffer containing 68 mM $(NH_4)_2SO_4$, 1 mg/ml BSA, 0.1% Tween-80, and 2.5 mM $MgCl_2$. Tubes with this mixture were incubated at 95°C for 5 min and rapidly cooled in an ice–water bath. The mixture was supplemented with 0.2 μM of each dNTP and 1 unit of *Taq* DNA polymerase. The reaction products were analyzed by electrophoresis in 1% agarose gel with the DNA of the methanotrophic bacteria *Methylobacter bovis* 98, *Methylomonas methanica* S1, *Methylomicrobium album* BG8, *Methylococcus capsulatus* Bath, *Methylosinus trichosporium* OB3b, and *Methylocystis parvus* OBBP as the control.

DNA–DNA hybridization was carried out on nylon filters (Hiiu Kalur, Estonia) [8] with [1',2',5'-^{3}H]deoxycytidine triphosphate and an enzyme kit for nick translation purchased from Amersham (United Kingdom). The reference strains of methylobacteria were *Methylobacterium extorquens* NCIMB 9399^T and *Paracoccus denitrificans* ATCC 17741^T. The reference methanotroph was *Methylocystis echinoides* 2 VKM B-2128^T.

The PCR products of the *pmoA* gene were purified on low-melting-point agarose with the use of agarase according to the manufacturer's instructions (Fermentas, Lithuania). The PCR fragment of the *pmoA* gene was sequenced with an automatic CEQ2000 XL sequenator (Beckman Coulter, United States) by using a CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter, United States). The preliminary phylogenetic screening of the nucleotide sequences and the translated amino acid sequences of the *pmoA* gene over the GenBank (NCBI) database was carried out with the aid of the BLAST program package (http://ncbi.nlm.nih.gov). The nucleotide sequences were translated into the amino acid sequences with the ORF Finder program (http://ncbi.nlm.nih.gov/gorf/gorf.html). To refine the phylogenetic position of the strains under study, the nucleotide and translated amino acid sequences of the *pmoA* gene were aligned manually (CLUSTAL W program at http://www.genebee.msu.su/clustal) with the latest relevant sequences available in the NCBI Database Project.

A rooted phylogenetic tree based on the translated amino acid sequences was constructed by the neighbor-joining method (NEIGHBOR) with the TREECON algorithm [17] and *Nitrosococcus oceanus* as the outgroup. Evolutionary distances were calculated as the number of substitutions per 100 amino acid residues. To assess the reliability of branching points on the tree, the sequence data were subjected to bootstrap analysis with the aid of the TREECON program. Bootstrap values were expressed as a percentage of 100 replications.

RESULTS

Good bacterial growth was observed in all the media with methanol that were inoculated with samples of the tree buds, seeds, and needles. The enrichment cultures had a deep pink color, typical of PPFM. The analysis of these cultures by the method of exhausting plating onto

Table 1. Some properties of methylobacteria isolated on methanol-containing media from different plant samples collected over the winter period

Source	Isolate	Pathway of C_1 metabolism	DNA–DNA homology (%) with	
			M. extorquens NCIMB 9399^T	*P. denitrificans* ATCC 17741^T
Linden buds	PPFM	Serine pathway	41	7
	Nonpigmented cocci	RuBP pathway	5	28
Linden seeds	PPFM	Serine pathway	42	7
	Nonpigmented cocci	RuBP pathway	6	26
Lilac buds	PPFM	Serine pathway	22	8
	Nonpigmented cocci	RuBP pathway	4	38
Apple buds	PPFM	Serine pathway	63	7
Maple buds	PPFM	Serine pathway	29	7
Pine needles	PPFM	Serine pathway	51	6
	Nonpigmented cocci	RuBP pathway	7	27
Spruce needles	PFM	Serine pathway	34	5

Note: PPFM, pink-pigmented facultative methylobacteria; RuBP, ribulose bisphosphate.

Table 2. Some characteristics of methanotrophic bacteria isolated from woody plant samples

Characteristics	Strain PL, isolated from linden buds	Strain GE, isolated from spruce needles
Morphology of vegetative cells	Slightly curved rods	
Intracytoplasmic membrane morphotype	II	II
Motility	+	+
Capsule	+	+
Resting form	Lipid cysts	
Growth on 0.1% methanol	+	+
Enzyme activity, nmol/(min mg protein):		
Hydroxypyruvate reductase, NADH	140	115
Hydroxypyruvate reductase, NADPH	95	84
Serine–glyoxylate aminotransferase, NADH	86	82
Serine–glyoxylate aminotransferase, NADPH	81	74
3-Hexulose-6-phosphate synthase	0	0
Ribulose bisphosphate carboxylase	0	0
Phosphoribulokinase	0	0
Isocitrate dehydrogenase, NAD^+	0	0
Isocitrate dehydrogenase, $NADP^+$	26	24
DNA–DNA homology (%) with *Methylocystis echinoides*	34	30

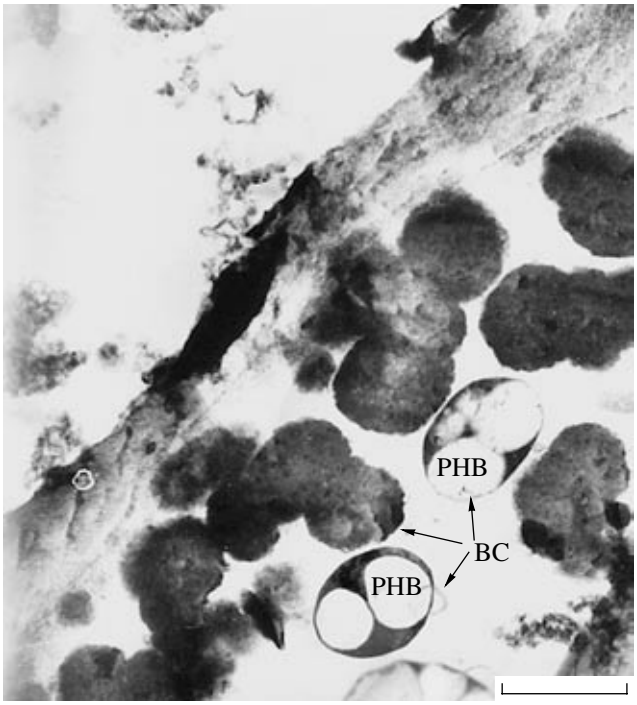

Fig. 1. Thin section of a blue spruce needle: BC, bacterial cell; PHB, polyhydroxybutyrate granule. The scale bar represents 1 μm.

agar media with methanol showed that they contained not only PPFM but also nonpigmented bacteria (in a proportion of approximately 2 : 1). The enrichments incubated under a CH_4/O_2 atmosphere showed good bacterial growth when the medium was inoculated with linden buds (association PL) or blue spruce needles (association GE).

PPFM were isolated in pure cultures from all the plant samples used. Nonpigmented methylobacteria were isolated from linden buds and seeds, lilac buds, and pine needles. Two methanotrophs were isolated from linden buds and blue spruce needles (Tables 1, 2). It should be noted that the thin sections of blue spruce needles exhibited the presence of bacterial cells (Fig. 1), some of which contained polyhydroxybutyrate (PHB) granules, typical of PPFM.

All PPFM isolates were similar in morphology (gram-negative motile and nonmotile rods of an irregular form) and had active key enzymes of the serine pathway of C_1 metabolism: hydroxypyruvate reductase (180–290 nmol/(min mg protein)) and serine–glyoxylate aminotransferase (120–170 nmol/(min mg protein)). They also accumulated PHB granules and exhibited a high level of DNA–DNA homology (22–63%) with *Methylobacterium extorquens* NCIMB 9399^T. All this allows the isolates to be ascribed to the genus *Methylobacterium*.

Figures 2 and 3 illustrate the morphology of bacteria isolated from linden buds and blue spruce needles, respectively. The nonpigmented, facultatively methy-

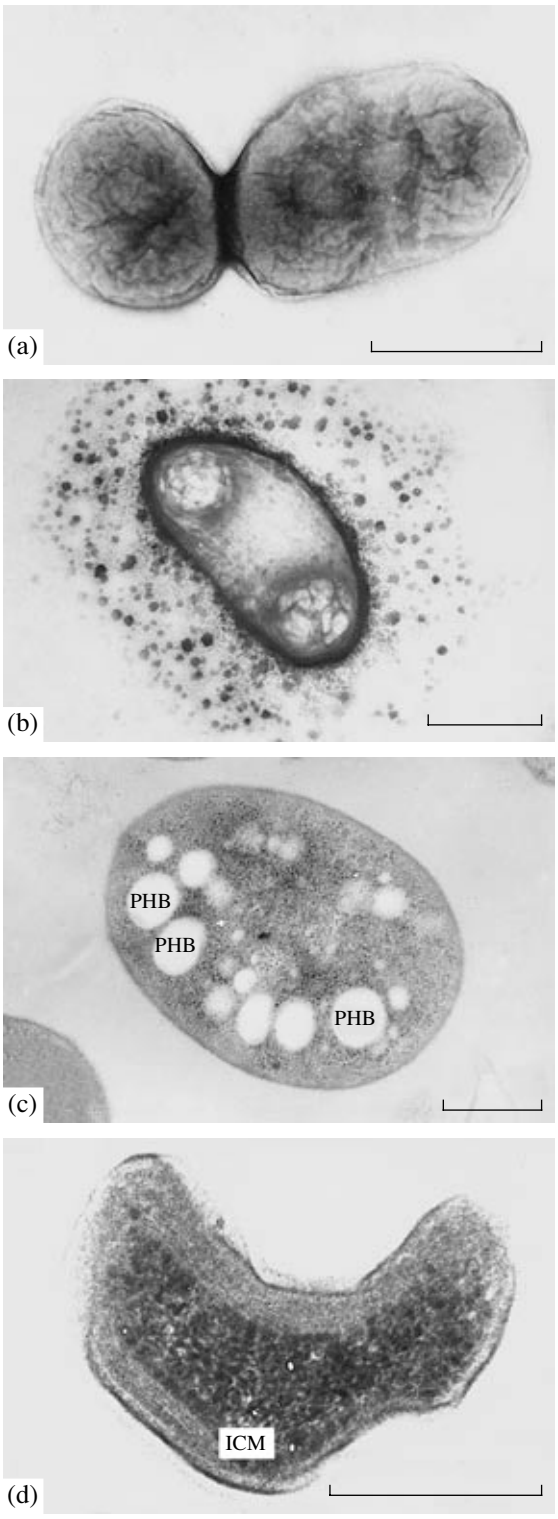

Fig. 2. The morphology of bacteria isolated from linden buds: (a) *Paracoccus* sp., negative staining; (b) *Methylobacterium* sp., negative staining; (c) *Methylobacterium* sp., thin section; (d) *Methylocystis* sp. PL, thin section. PHB is polyhydroxybutyrate; ICM is intracytoplasmic membrane. The scale bars represent 0.5 μm.

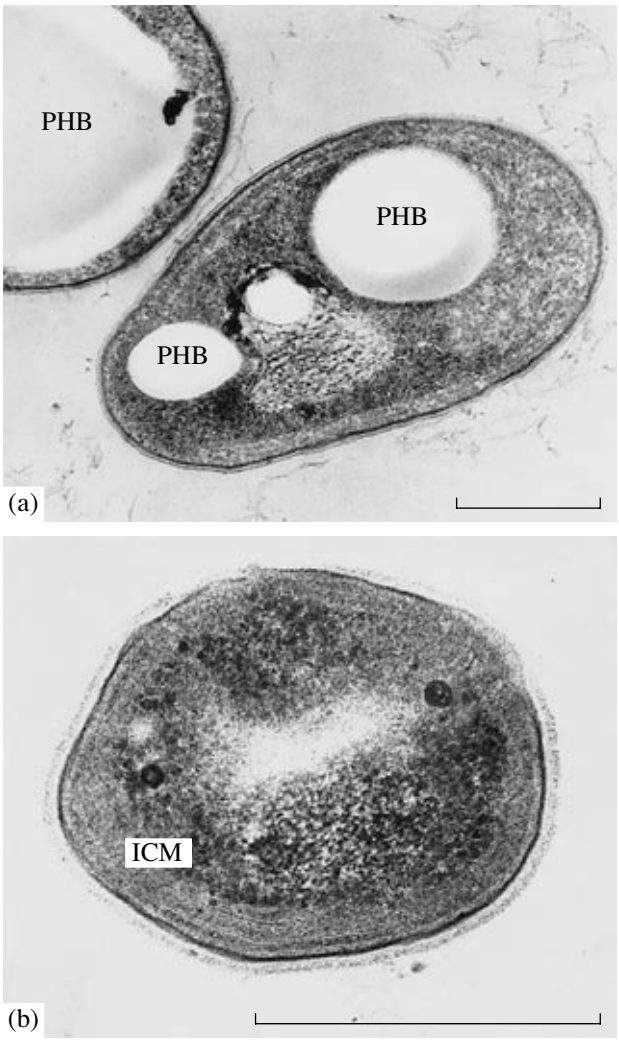

Fig. 3. Thin sections of methylotrophic bacteria isolated from blue spruce needles: (a) *Methylobacterium* sp.; (b) *Methylocystis* sp. GE. PHB is polyhydroxybutyrate; ICM is intracytoplasmic membrane. The scale bars represent 0.5 μm.

lotrophic, gram-negative, nonmotile, coccoid bacteria isolated from linden seeds and buds, lilac buds, and pine needles possessed active hydroxypyruvate reductase (40–90 nmol/(min mg protein)) and the key enzymes of the ribulose bisphosphate pathway (phosphoribulokinase and ribulose bisphosphate carboxylase) with activities of 70–85 and 120–160 nmol/(min mg protein), respectively. At the same time, these isolates did not contain serine–glyoxylate aminotransferase or 3-hexulose-6-phosphate synthase (the key enzyme of the ribulose monophosphate pathway). In the degree of DNA–DNA homology (26–38%), the isolates were close to *Paracoccus denitrificans* ATCC 17741^T. These data suggest that the isolates are close to methylobacteria of the genus *Paracoccus*.

The PCR analysis of the DNA of the methanotrophic cultures PL and GE with the primers specific to the genera *Methylosinus/Methylocystis* gave rise to a product with an expected length. In contrast, PCR analysis with the primers specific to the genera *Methylomonas*, *Methylobacter, Methylococcus, Methylomicrobium*, and *Methylosinus* did not give the expected products (Table 3).

Methanotrophs in natural samples are often detected with the aid of the *pmoA* gene, which is present in all the methanotrophs known so far except for representatives of the genus *Methylocella* (they contain only soluble methane monooxygenase) [18]. The results of the phylogenetic analysis of the nucleotide sequence and the translated amino acid sequence of a given region of the *pmoA* gene correlate with the data of the phylogenetic analysis of 16S rRNA, which allows the *pmoA* gene sequences to be used for the identification of bacterial isolates to the species level [19]. The PCR analysis of the total DNA of the methanotrophic enrichments for the presence of the *pmoA* and *mmoX* genes, which encode, respectively, particulate and soluble methane monooxygenase, showed the presence of *pmoA* alone. A comparison of the nucleotide sequences and the translated amino acid sequences of the *pmoA* genes of pure methanotrophic cultures (strains PL and GE) and the representatives of the genus *Methylocystis* showed a high level of their relatedness (95.7–98% similarity of the amino acid sequences and 93.3–97.7% similarity of the nucleotide sequences).

The thin sections of methanotrophic cells isolated from linden buds and blue spruce needles showed the presence of intracytoplasmic membranes of morphotype II (Figs. 2d, 3b). Both methanotrophic strains possessed the indicator enzymes of the serine pathway and $NADP^+$-linked isocitrate dehydrogenase (Table 2). PCR analysis with the primers specific to the genera *Methylosinus/Methylocystis* gave rise to a product with an expected length. In contrast, PCR analysis with the primers specific to the genus *Methylosinus* failed to reveal the expected products. The results of the phylogenetic analysis of the *pmoA* gene (Fig. 4) and the data of DNA–DNA hybridization with the type strain *Methylocystis echinoides* 2 (Table 2) give grounds to believe that the methanotrophs isolated from plant tissues belong to the genus *Methylocystis*.

Thus, all the samples of tree seeds, buds, and needles collected over the winter period were found to contain methylotrophic bacteria.

DISCUSSION

Based on the results of experiments with corn, Romanovskaya *et al.* [6] concluded that the phyllosphere-inhabiting PPFM are carried to soil with leaves during the fall and again colonize new leaves in the spring, being carried from the soil by airborne soil particles. This conclusion, however, contradicts the results of experiments with a labeled PPFM strain, which, when introduced into the soil, did not colonize the new

Table 3. The PCR analysis of the total DNA of methanotrophic enrichment cultures for the presence of the sMMO and pMMO genes and the 16S rRNA of some reference methanotrophs

Primer	Sequence	Ref.	Specificity	Total DNA of enrichments	
				Linden buds (strain PL)	Spruce needles (strain GL)
pmoA189f/	GGNGACTGGGACTTCTGG	[13]	pMMO	+	+
mb 661r	CCGGMGCAACGTCYTTACC	[13]			
mmoX2008f/	CGGTCCGCTGTGGAAGGGCATGAAGCGCGT	[14]	sMMO	–	–
mmoX2376r	GGCTCGACCTTGAACTTGGAGCCATACTCG	[14]			
16rRNA27f/	AGAGTTTGATCMTGGCTCAG	[15]			
Mb 884r	ATGCGTTCTGCGCCACTA	[15]	*Methylobacter*	–	–
Mm 1007r	CACTCCGCTATCTCTAACAG	[15]	*Methylobacter*/ *Methylomonas*	–	–
Mb 1007r	CACTCTACGATCTCTCACAG	[15]	*Methylomicrobium*	–	–
Mlc 1436	CCCTCCTTGCGGTTAGACTACCTA	[16]	*Methylococcus*	–	–
Ms 1020r	CCCTTGCGGAAGGAAGTC	[15]	*Methylosinus*	–	–
Type 2b	CATACCGGRCATGTCAAAAGC	[13]	*Methylosinus*/ *Methylocystis*	+	+

Note: pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase.

leaves of corn seedlings. Moreover, even if PPFM seasonally colonize the phyllosphere of corn plants, this fact cannot be generalized to all plants, including perennial and woody ones. Despite the evidence that PPFM are associated with many plants [1], we were able to isolate from the corn phyllosphere and rhizosphere predominantly nonpigmented methylobacteria belonging to the genera *Methylovorus* [8] and *Paracoccus* [9]. This can be accounted for by the fact that corn (a plant with the C_4-type metabolism) is predominantly associated with methylobacteria other than PPFM.

All the samples of deciduous and coniferous tree tissues collected over the winter period and plated on selective media were found to contain methylotrophic bacteria. It should be noted, however, that the leaves collected over the summer period gave rise to a rapid growth of PPFM on the agar surface below the leaves, whereas the buds, seeds, and needles collected over the

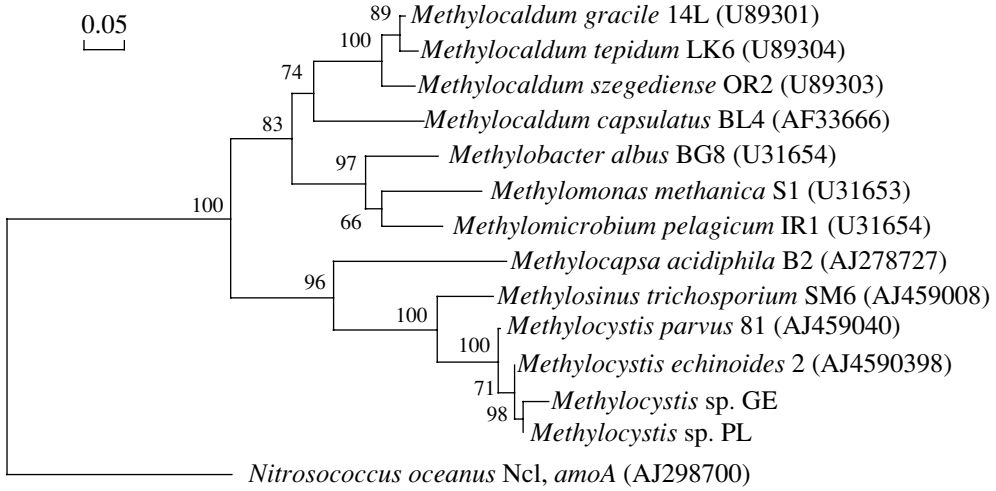

Fig. 4. A phylogenetic tree constructed on the basis of a comparative analysis of the PmoA fragments of the methanotrophic GE and PL isolates and the analogous PmoA fragments of some methanotrophic bacteria of types I and II. The tree was rooted with the *amoA* gene sequence of *Nitrosococcus oceanus* as the outgroup. The scale bar represents 5 amino acid substitutions per 100 amino acids. The numerals at the branching points are bootstrap values from 100 replicates.

winter period gave rise to PPFM cells growing on the agar surface only after a long-term incubation of the plates in a humid atmosphere in the presence of methanol. These results suggest the occurrence of methylobacteria inside plant tissues. The electron microscopic examination of the thin sections of blue spruce needles confirmed that they contain methylobacteria inside.

PCR analysis indicated the presence of methanotrophs in two enrichment cultures, which were obtained from the samples of linden buds and blue spruce needles. These methanotrophs were then isolated in pure cultures. The isolates were found to belong to the genus *Methylocystis*; to use the serine pathway of C_1 metabolism; to have type II intracytoplasmic membranes; and to form lipid cysts highly resistant to heating at 70°C for 20 min and desiccation, i.e., to temperature and humidity stresses. It should be noted that representatives of the genus *Methylocystis* have so far been isolated only from soil, freshwater, and marine environments [10].

The data obtained suggest that aerobic methylotrophic bacteria are permanently associated with plants. In the winter, when plant metabolism is suppressed, these bacteria predominantly occur inside plant tissues. Being immobilized there, they can well survive repeated freezing–thawing cycles [20], extreme temperatures, and drought. Under favorable ambient conditions, when plants actively grow and excrete volatile C_1 compounds, methylotrophic bacteria produce biofilms on the leaf surface, which prevent the evaporation of the volatile compounds into the atmosphere.

There is ever-increasing evidence that aerobic methylotrophic bacteria are symbiotically associated with plants [1]. Some bioactive compounds that are excreted by the live cells of these bacteria or appear in the medium because of cell lysis may exert beneficial effects on the growth and development of plants. In addition, some methylotrophic bacteria, morphotype II methanotrophs in particular, are able to fix dinitrogen [10]. This allows the suggestion to be made that the occurrence of methylotrophic bacteria not only on the surface of but also inside plant tissues is beneficial for phytosymbiosis. The data presented in this paper provide further evidence that the plant phyllosphere is colonized by methylotrophs during the vegetative period mainly due to the development of bacterial cells occurring permanently inside plant tissues. Work is now in progress in our laboratory on investigating the taxonomic status of the isolated methanotrophs and methylobacteria and studying the structural and functional fundamentals of their interaction with woody plants.

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