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EXPERIMENTAL ARTICLES

Methylovorus mays sp. nov.: A New Species of Aerobic, Obligately Methylotrophic Bacteria Associated with Plants

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Abstract—A bacterial strain utilizing methanol as the sole source of carbon and energy was isolated from the maize phyllosphere. Cells are nonpigmented gram-negative motile rods that do not form spores or prosthecae and reproduce by binary fission. The strain does not require vitamins or supplementary growth factors. It is obligately aerobic and urease-, oxidase-, and catalase-positive. The optimum growth temperature is 35–40°C; the optimum pH is 7.0–7.5. The doubling time is 2 h. The bacterium implements the ribulose monophosphate pathway and possesses NAD^+-dependent 6-phosphogluconate dehydrogenase and enzymes of the glutamate cycle. α-Ketoglutarate dehydrogenase and enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) are absent. Fatty acids are dominated by palmitic ($C_{16:0}$) and palmitoleic ($C_{16:1}$) acids. The major phospholipids are phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine. Cardiolipin is present in minor amounts. The dominant ubiquinone is Q_8. The bacterial genome contains genes controlling the synthesis and secretion of cytokinins. The G+C content of DNA is 57.2 mol %, as determined from the DNA thermal denaturation temperature (T_m). The bacterium shows low DNA homology (<10%) with restricted facultative methylotrophic bacteria of the genus *Methylophilus* (*M. methylotrophus* NCIMB 10515^T and *M. leisingerii* VKM B-2013^T) and with the obligate methylotrophic bacterium (*Methylobacillus glycogenes* ATCC 29475^T). DNA homology with the type representative of the genus *Methylovorus*, *M. glucosetrophus* VKM B-1745^T, is high (58%). The new isolate was classified as a new species, *Methylovorus mays* sp. nov.

Key words: *Methylovorus mays*, obligate methylotroph, ribulose monophosphate pathway, cytokinins

The finding that most plants produce and excrete methanol [1] led to the idea of methanol cycling between plants, methylotrophs, and the atmosphere [2]. Methanol excretion by plants is regulated by the permeability of leaf stomata. There is experimental evidence that methanol excreted by plants can be utilized by aerobic methylotrophic bacteria inhabiting the leaf surface.

Some authors emphasized the existence of a relationship between pink-pigmented facultative methylotrophs (PPFMs) and plants [3–5]. Our investigation of the microflora of eleven plant species showed that such a relationship is typical of not only PPFMs but also other aerobic methylobacteria [6], which are involved in the biogeochemical cycle of methanol by utilizing it as a growth substrate [2]. Aerobic methylobacteria are presumably phytosymbionts rather than commensals. This follows from the finding of Long *et al.* [7], who, using the polymerase chain reaction technique, showed the presence in a PPFM of the gene of dimethylallyl transferase, the enzyme responsible for the first step of cytokinin biosynthesis.

The present work aimed at studying and identifying a novel strain of obligately methylotrophic bacteria associated with maize.

MATERIALS AND METHODS

Strain BV was isolated from leaves of the maize *Zea mays* L. using agar medium K [8] with 0.5 vol % methanol. As reference cultures, we used the following type strains of methylotrophic bacteria: *Methylophilus methylotrophus* VKM B-1623^T (= NCIMB 10515), *Methylophilus leisingerii* VKM B-2013^T), *Methylobacillus glycogenes* VKM B-2060^T (= ATCC 29475), *Methylovorus glucosetrophus* VKM B-1745^T (ATCC 49758), and *Methylophaga marina* VKM B-2056 (ATCC 35842). Bacteria were grown in 750-ml flasks with 200 ml of medium K containing 0.5% methanol on a shaker (180 rpm) at 29 or 37°C.

The investigation of cultural, morphological, physiological, and biochemical characteristics, the determination of ubiquinones, fatty acids, phospholipids, and enzyme activities, as well as the PCR-based analysis of genes controlling the synthesis and secretion of cytokinins were performed as described earlier [4, 8].

DNA was isolated and purified by the method of Marmur [9]. The G+C content of DNA was determined by the thermal denaturation method on a Pye Unicam SP 1800 spectrophotometer at a heating rate of 0.5 deg/min. The degree of DNA–DNA homology was evaluated from DNA reassociation rates [10].

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Table 1. Activities of enzymes of the primary and intermediary metabolism of methanol in extracts of strain BV cells

Enzyme	Cofactor	Activity, nmol/(min mg protein)
Methanol dehydrogenase	PMS	125
Formaldehyde dehydrogenase	PMS	14
	NAD^+	0
Formate dehydrogenase	PMS	10
	NAD^+	0
Hexulose-3-phosphate synthase		140
Glucose-6-phosphate dehydrogenase	NAD^+	310
	$NADP^+$	360
6-Phosphogluconate dehydrogenase	NAD^+	97
	$NADP^+$	0
Fructose-1,6-bisphosphate aldolase		0
2-Keto-3-deoxy-6-phosphogluconate aldolase		46
Hydroxypyruvate reductase	NAD(P)H	0
Serine–glyoxylate aminotransferase	NADH	0
Ribulose 1,5-bisphosphate carboxylase		0
Isocitrate dehydrogenase	NAD^+	120
	$NADP^+$	40
α-Ketoglutarate dehydrogenase	NAD^+	0
Malate synthase		0
Isocitrate lyase		0
Glutamate dehydrogenase	NAD(P)H	0
Glutamate synthase	NAD(P)H	28
Glutamine synthetase	Mn^{2+}, ATP	34

RESULTS

The obtainment of enrichment and pure cultures. In order to obtain enrichment cultures, samples of leaves, stem, and roots were aseptically cut from a young maize plant (about 25 cm in height) picked in a field in the Moscow region and incubated in medium K with 0.5% methanol. Good growth of nonpigmented methylotrophic bacteria was observed in the media inoculated with samples of maize phyllosphere and rhizosphere. Pure bacterial cultures obtained from some single colonies grown on agar medium were identical and represented obligately methylotrophic bacteria. For further studies, we chosed strain BV isolated from maize leaves, where its population ranged from 90 to 600 CFU/cm^2.

Morphology. The cells of the isolate were gram-negative asporogenous motile monotrichous rods 0.4–0.6 by 1.1–1.4 μm in size. They reproduced by binary fission and did not possess an intracytoplasmic membrane system.

Cultural, physiological, and biochemical properties. The isolate grew well in a liquid medium with methanol, without forming cell aggregates or producing pigments. The bacterium was obligately aerobic and did not require vitamins. The doubling time was 2 h. Colonies grown on agar medium with 0.5% methanol at 35°C for 96 h were circular, translucent, colorless, with an entire edge and a smooth glossy surface, up to 1 mm in diameter. The bacterium was obligately methylotrophic: it did not grow on methane, methylated amines, organic acids, alcohols, carbohydrates, amino acids, $H_2 + O_2 + CO_2$ mixture, nutrient and wort agar.

The bacterium failed to hydrolyze cellulose, gelatin, starch, and milk. Reactions for oxidase, catalase, and urease were positive. Nitrates were reduced to nitrites. Sugars were not fermented. The lipase, methyl red, and Voges–Proskauer tests were negative. The bacterium could utilize ammonium salts, nitrates, and some amino acids as nitrogen sources. In a medium with 0.5% methanol and nitrate as the nitrogen source, the isolate produced indole from 1% tryptophan.

The bacterium could grow at temperatures between 20 and 45°C and at pH values between 6.5 and 8.5. Optimum growth temperature and pH were 35–40°C and 7.0–7.5, respectively. Growth was inhibited by 3% NaCl. The isolate was sensitive to gentamicin, kanamycin, nalidixic acid, neomycin, novobiocin, streptomycin, and erythromycin and resistant to ampicillin and

lincomycin (all antibiotics were taken in the amount of 30 μg/disk).

Metabolic characteristics. The activities of enzymes of the primary and intermediary metabolism of methanol in cell extracts of strain BV are shown in Table 1. It can be seen that the cells contained the phenazine methosulfate–dependent dehydrogenases of methanol, formaldehyde, and formate, hexulose-3-phosphate synthase (the key enzyme of the ribulose monophosphate (RuMP) pathway), NAD^+- and $NADP^+$-dependent glucose-6-phosphate dehydrogenases, NAD^+-dependent 6-phosphogluconate dehydrogenase, 2-keto-3-deoxy-6-phosphogluconate aldolase, NAD^+- and $NADP^+$-dependent isocitrate dehydrogenases, and enzymes of the glutamate cycle (glutamate synthase and glutamine synthetase). At the same time, we were unable to reveal $NADP^+$-dependent 6-phosphogluconate dehydrogenase, α-ketoglutarate dehydrogenase, glutamate dehydrogenase, and specific enzymes of the serine pathway (hydroxypyruvate reductase and serine–glyoxylate aminotransferase), the Calvin cycle (ribulose 1,5-bisphosphate carboxylase), and the glyoxylate cycle (malate synthase and isocitrate lyase). These data suggest that strain BV implements the 2-keto-3-deoxy-6-phosphogluconate modification of the RuMP pathway and that ammonium is assimilated via the glutamate cycle.

Chemo- and genotaxonomic characteristics. The fatty acids of strain BV were dominated by palmitic ($C_{16:0}$) and palmitoleic ($C_{16:1}$) acids (Table 2). The major phospholipids were phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine. Cardiolipin was detected in minor amounts. The dominant ubiquinone was Q_8.

The G+C content of DNA was 57.2 mol %, as determined from the DNA thermal denaturation temperature (T_m). DNA homology with the type representatives of the genera *Methylophilus, Methylobacillus*, and *Methylophaga* was low (less than 8%), but it was high with the type representative of the genus *Methylovorus* (56–58%). The bacterial genome contained genes controlling the synthesis and secretion of cytokinins.

Table 2. Fatty acid profile of strain BV cells

Fatty acid	% of total content
$C_{14:0}$	0.85
$C_{15:0}$	0.90
cis-$C_{16:1}\omega$	34.15
trans-$C_{16:1}\omega7$	2.35
$C_{16:0}$	48.91
$\Delta C_{17:0}$	9.74
$C_{17:0}$	0.32
cis-$C_{18:1}\omega9$	0.20
cis-$C_{18:1}\omega7$	1.69
trans-$C_{18:1}\omega7$	0.10
$C_{18:0}$	0.69
$\Delta C_{19:0}$	0.10

DISCUSSION

It is known that pink-pigmented facultatively methylotrophic representatives of the genus *Methylobacterium* with the serine pathway of C_1-metabolism are permanent inhabitants of the plant phyllosphere [3–5]. Strain BV, isolated by us from maize leaves, is the first nonpigmented obligate methylotroph implementing the RuMP pathway. Four genera of obligate and restricted facultative methylotrophic bacteria with the RuMP pathway—*Methylophilus, Methylobacillus, Methylophaga*, and *Methylovorus*—are presently known [11–14]. Obligate methylotrophic bacteria are difficult to differentiate using routine morphological, cultural, and physiological criteria, since their species are similar in cell morphology, and obligate methylotrophy restricts the use of standard physiological and biochemical tests. Restricted facultative methylotrophic bacteria are similar to obligate methylotrophic bacteria in physiological and biochemical properties but differ from them in the ability to utilize, additionally to C_1-compounds, glucose or fructose [11, 13, 14].

Obligate and restricted facultative methylotrophic bacteria with the RuMP pathway are also similar in some chemotaxonomic characteristics: their fatty acids are dominated by palmitic acid (saturated $C_{16:0}$ acid with an unbranched side chain) and palmitoleic acid (unsaturated $C_{16:1}$ acid), and their major ubiquinone is Q_8. At the same time, bacteria of the genus *Methylophilus* differ from the representatives of other related genera in the fact that they contain no diphosphatidylglycerol (cardiolipin). The genus *Methylophaga* was proposed for marine methylotrophic bacteria, which have a low G+C content of DNA (38–46 mol %), are moderately halophilic, and auxotrophic for vitamin B_{12}.

Like other known obligate and restricted facultative methylotrophic bacteria with the RuMP pathway [15, 16], strain BV did not possess enzymes necessary for heterotrophy (by the way, this explains the obligate methylotrophy of this strain).

The isolate considerably differed from bacteria of the genus *Methylophaga* by a higher G+C content of DNA, lower tolerance to NaCl, absence of enzymes responsible for the oxidation of methylated amines, and lack of requirement for vitamin B_{12}. The degree of DNA–DNA homology between strain BV and members of the genus *Methylophaga* was low.

Strain BV also differed from obligate methylotrophic bacteria of the genus *Methylobacillus*: it had a different G+C content of DNA and lacked glutamate dehydrogenase and $NADP^+$-dependent 6-phosphogluconate dehydrogenase. The degree of DNA–DNA

Table 3. Differentiating characteristics of obligate and restricted facultative methylotrophic bacteria with the RuMP pathway

Characteristic	*Methylophilus*	*Methylobacillus*	*Methylophaga*	*Methylovorus*
G+C, mol %	50–53	50–56	38–46	56–57
Growth in the presence of 6% NaCl	–	–	+	–
Auxotrophy for B_{12}	–	–	+	–
Ammonium assimilation	Via the glutamate cycle	Via glutamate dehydrogenase	Via the glutamate cycle and glutamate dehydrogenase	Via the glutamate cycle
Isocitrate dehydrogenase (NAD^+)	+	+	+	–
Isocitrate dehydrogenase ($NADP^+$)	–	+	+	+
6-Phosphogluconate dehydrogenase ($NADP^+$)	+	+	+	–
Cardiolipin	–	+	+	+
Optimum temperature, °C	30–37	29–42	29–32	35–37

homology between the isolate and the type species *M. glycogenes* ATCC 29475^T was low.

Furthermore, the isolate differed from methylotrophic bacteria of the genus *Methylophilus* by a higher G+C content of DNA, the absence of $NADP^+$-dependent 6-phosphogluconate dehydrogenase, and the presence of cardiolipin. The degree of DNA–DNA homology between strain BV and the type species *M. methylotrophus* ATCC 10515^T was low.

It is believed that strains whose DNAs differ by more than 10% in the G+C content belong to different genera [17], and strains showing DNA–DNA hybridization values lower than 70% belong to different species [18]. Based on the values of these parameters and on the data of comparative chemo- and genotaxonomic analyses, we assigned strain BV to the genus *Methylovorus* (Table 3). At the same time, the isolate differed from the type representative of this genus, *M. glucosetrophus* VKM B-1745^T, by its inability to utilize glucose as the source of carbon and energy or to hydrolyze starch. The degree of DNA–DNA homology between strains BV and B-1745^T was low (56–58%). Therefore, strain BV may be considered a new species of the genus *Methylovorus*, which was named by us *Methylovorus mays*.

Strain BV cannot be an accidental inhabitant of the maize phyllosphere and rhizosphere: being obligately dependent on the methanol produced by maize plants, this methylotroph may provide plants with cytokinins, as it contains genes responsible for the synthesis and secretion of these phytohormones. Below, a taxonomic description of *Methylovorus mays* sp. nov. is given.

Methylovorus mays (ma'ys. L. adj. *mays*, referring to old Sp. n. *maiz*, maize) was named for its host plant, the maize *Zea mays* L. Cells are gram-negative, nonpigmented, asporogenous rods 0.4–0.6 by 1.1–1.4 μm in size, motile by means of one polar flagellum. Colonies grown on methanol-containing agar medium after 3-day incubation at 35°C are circular, with an entire edge and smooth surface, convex, 1 mm in diameter, translucent, colorless or pinkish. Growth occurs in a temperature range of 20–45°C at pH 6.5–8.5; optimum growth occurs at 35–40°C and pH 7.0–7.5. The doubling time is 2 h. Requires no vitamins or supplementary growth factors. Cellulose, gelatin, and starch are not hydrolyzed. Acetoin, hydrogen sulfide, and ammonia are not produced. Oxidase-, catalase-, and urease-positive. Obligately aerobic. Reduces nitrates to nitrites. Utilizes methanol as the sole source of carbon and energy. Utilizes ammonium salts, nitrates, and some amino acids as nitrogen sources. Produces indole from 1% tryptophan in the growth medium with 0.5% methanol as the carbon source and nitrate as the nitrogen source. Growth is inhibited by 3% NaCl. Implements the 2-keto-3-deoxy-6-phosphogluconate variant of the RuMP pathway. The tricarboxylic acid cycle is open at the level of α-ketoglutarate dehydrogenase. Enzymes of the glyoxylate cycle (malate synthase and isocitrate lyase) are absent. Ammonium is assimilated via the glutamate cycle. Fatty acids are dominated by palmitic ($C_{16:0}$) and palmitoleic ($C_{16:1}$) acids. The major phospholipids are phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine. Cardiolipin is present in minor amounts. The dominant ubiquinone is Q_8. The G+C content of DNA is 57.2 mol % (T_m). The type strain *M. mays* BV was isolated from leaves of a maize plant picked in the Moscow region and is deposited in the All-Russia Collection of Microorganisms as strain VKM B-2221.

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