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EXPERIMENTAL ARTICLES

The Ways of Plant Colonization by *Methylobacterium* Strains and Properties of These Bacteria

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Abstract—The pink-pigmented facultative methylotrophic bacteria (PPFMB) of the genus *Methylobacterium* are indispensible inhabitants of the plant phyllosphere. Using maize *Zea mays* as a model, the ways of plant colonization by PPFMB and some properties of the latter that might be beneficial to plants were studied. A marked strain, *Methylobacterium mesophilicum* APR-8 (pULB113), was generated to facilitate the detection of the methylotrophic bacteria inoculated into the soil or applied to the maize leaves. Colonization of maize leaves by *M. mesophilicum* APR-8 (pULB113) occurred only after the bacteria were applied onto the leaf surface. In this case, the number of PPFMB cells on inoculated leaves increased with plant growth. During seed germination, no colonization of maize leaves with *M. mesophilicum* cells occurred immediately from the soil inoculated with the marked strain. Thus, under natural conditions, colonization of plant leaves with PPFMB seems to occur via soil particle transfer to the leaves by air. PPFMB monocultures were not antagonistic to phytopathogenic bacteria. However, mixed cultures of epiphytic bacteria containing *Methylobacterium mesophilicum* or *M. extorquens* did exhibit an antagonistic effect against the phytopathogenic bacteria studied (*Xanthomonas campestris, Pseudomonas syringae, Erwinia carotovora, Clavibacter michiganense,* and *Agrobacterium tumifaciens*). Neither epiphytic nor soil strains of *Methylobacterium extorquens, M. organophillum, M. mesophilicum,* and *M. fujisawaense* catalyzed ice nucleation. Hence, they cause no frost injury to plants. Thus, the results indicate that the strains of the genus *Methylobacterium* can protect plants against adverse environmental factors.

Key words: *Methylobacterium*, plant colonization, ice crystallization, antagonistic properties.

Pink-pigmented facultative methylotrophic bacteria (PPFMB) of the genus *Methylobacterium* are persistently present in the phyllosphere of various plants [1–4]. They were revealed on leaves of almost all the plants, whereas no PPFMB were found in the branch, core, and leaf buds [4]. Successful coexistence of PPFMB with plants is largely due to their ability to assimilate methanol, which is a plant exometabolite [3]. The aim of the present work was to elucidate the ways of plant colonization by PPFMB and to study some of their properties that are beneficial to plants.

MATERIALS AND METHODS

Bacteria. The epiphytic and soil strains of PPFMB *Methylobacterium mesophilicum, M. extorquens,* and *M. fujisawaense* were isolated from various ecosystems and regions of the Ukraine [4]. These strains are listed in Table 1.

Cultivation conditions. *Methylobacterium* strains were grown on mineral medium [4], either liquid or agarized (MM and MMA, respectively), containing methanol (0.5 vol %) as the only carbon source and on standard glucose–potato agar (GPA). Phytopathogenic bacteria were cultivated on GPA, whereas the obligate methane-utilizing bacteria were cultivated on methanol-free MM or MMA in the presence of methane as the only carbon source [5]. All strains studied were grown at 30°C and pH 7.0.

Generation of the marked strain. The marked strain was obtained by conjugal transfer of a plasmid from the *Methylomonas rubra* 15sh (pULB113) [6] to *Methylobacterium mesophilicum* 4181(Str^R). The conjugative plasmid pULB113 had been previously provided from the Laboratory of the Methylotrophic Microorganism Genetics (Research Institute of Genetics, Moscow) to construct the strain *Methylomonas rubra* 15sh (pULB113) [6]. pULB113 is a broad-host-range plasmid which confers the resistance to kanamycin, tetracycline, and ampicillin. The plasmid conjugal transfer was conducted as described by Warner *et al.* [7]. A single colony of the *Methylomonas rubra* 15sh (pULB113) donor grown on agarized medium in the presence of methane was mixed in a spot with a colony of the recipient *Methylobacterium mesophilicum* 4181(Str^R) on a dish containing nonselective MMA

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Table 1. Methylotrophic bacteria used in this study

Species	Strain designation		Source	
	IMV collection	laboratory	region	ecosystem
Methylobacterium extorquens	3351	K2	The city of Kiev	Cactus leaves (*Cactus* sp.)
The same	3360	LKL	Odessa oblast	Clover leaves (*Trifolium pratense*)
″	3369	B121	Suburb of Kiev	Grape leaves (*Vitis omerensis*)
Methylobacterium mesophilicum	3337	8–18	Zone of CNPP	Soddy-podzolic soil; depth, 8–10 cm
The same	3352	Ch8	Suburb of Kiev	Celandine leaves (*Chelidonium* sp.)
″	3354	D10	The same	Clover leaves (*Trifolium pratense*)
″	3380	4181	Obtained from ATCC (ATCC 29983)	
″	3399	4181(Str^R)	Streptomycin-resistant mutant *Methylobacterium mesophilicum* 4181	
Methylobacterium fujisawaense	3365	B5	Suburb of Kiev	Grape leaves (*Vitis omerensis*)
The same	3370	B15	The same	Grape leaves (*Vitis omerensis*)
Methylobacterium sp.	3357	T2	Lugansk oblast	Poplar leaves (*Populus piramidalis*)
Methylomonas rubra	3128	15sh (pULB113)	Plasmid-carrying mutant *Methylomonas rubra* 15sh [6]	

Note: IMV is Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kiev; CNPP is Chernobyl Nuclear Power Plant; ATCC is American Type Culture Collection, Rockville, Md., United States.

medium with no antibiotics. After incubation at 30°C for 18 h, the cells were washed off with a physiologic saline (0.5% NaCl), and 0.1 ml of cell suspension was plated onto selective medium (MMA + 50 μg kanamycin/ml) to be incubated for five days. Selection of transconjugant colonies *M. mesophilicum* 4181(Str^R) and their growing proceeded on the selective medium.

To determine strain resistance to antibiotics, the bacterial suspensions of the transconjugants, the wild strain *Methylobacterium mesophilicum* 4181, and the mutant 4181(Str^R) (10^9 cells/ml) were plated on the dishes with MMA containing antibiotics at different concentrations: kanamycin, from 2 to 50 μg/ml; tetracycline, from 1 to 40 μg/ml; ampicillin, from 10 to 300 μg/ml; streptomycin, from 200 to 500 μg/ml.

Plant colonization with PPFMB. In model experiments, we used the marked PPFMB strain *Methylobacterium mesophilicum* APR-8 (pULB113) and the maize *Zea mays*, Kollektivnyi variety, no. 675-37, the seeds of which were provided by the Institute of Plant Physiology and Selection, National Academy of Sciences of Ukraine (see RESULTS AND DISCUSSION). The experiments were conducted in 300-cm^3 vegetation flasks under laboratory conditions (a continuous photoperiod, no less than 12 h; from 14 to 18°C night temperature; from 22 to 24°C day temperature; 75% relative humidity). To reveal other PPFMB strains in soil and seeds used for the experiments, samples were plated onto MMA medium containing methanol. The lack of the pink-pigmented colonies five to seven days after plating indicated that no PPFMB were present in the material studied.

Two possible ways of plant colonization by PPFMB were studied: from soil during seed germination and after direct application of bacteria onto plant leaves. Each experiment was repeated five times.

Plant colonization by PPFMB from soil. A suspension of the marked strain *M. mesophilicum* APR-8 (pULB113) grown on MMA was prepared in a 0.5% NaCl solution and introduced into nonsterile soil to a final concentration of 10^6 cells per g soil. The inoculated soil was mixed and transferred into ten 300-cm^3 experimental vegetation flasks. The control soil was supplemented with an equivalent volume of 0.5% NaCl, and, after mixing, placed into five flasks.

Nonsterile maize seeds were placed into dishes on moistened filter paper and germinated in a thermostat at 30°C for two days. After germination, the seeds were introduced at a depth of 1 cm into the soil contained either in the experimental flasks (soil inoculated by cells of the marked strain APR-8) or in the control flasks with noninoculated soil. Maize growth proceeded for 30 to 35 days.

To discern whether the maize colonization with APR-8 cells contained in soil did occur during seed germination, the surface of the maize leaves were tested for the presence of APR-8 cells. For this purpose, 1 cm^2

Table 2. Cell number of *M. mesophilicum* APR-8 in soil and on maize leaves (model experiments)

Variant	Sample studied	CFU of strain APR-8 in 1g of soil or on 1 cm^2 of leaf after maize growing for various time intervals (days)					
		0	8	12	16	20	30
Soil inoculated with APR-8 cells							
Seeds untreated with APR-8	Soil	$1.4 \times 10^6 \pm 1.2 \times 10^5$	$1.5 \times 10^6 \pm 1.0 \times 10^5$	$3.0 \times 10^6 \pm 1.1 \times 10^5$	$3.3 \times 10^6 \pm 2.1 \times 10^5$	$1.1 \times 10^5 \pm 2.0 \times 10^4$	$5.5 \times 10^4 \pm 2.3 \times 10^3$
The same	Leaves	0	0	0	0	0	0
Seeds after exposure to APR-8	Soil	$1.1 \times 10^6 \pm 1.2 \times 10^5$	$1.5 \times 10^6 \pm 2.3 \times 10^5$	$1.4 \times 10^5 \pm 1.8 \times 10^4$	$5.6 \times 10^6 \pm 3.2 \times 10^4$	$3.2 \times 10^5 \pm 1.4 \times 10^4$	$6.6 \times 10^4 \pm 1.7 \times 10^3$
The same	Leaves	0	0	0	0	0	0
Seeds and soil with no APR-8 added (control)	Soil	0	0	0	0	0	0
The same	Leaves	0	0	0	0	0	0
Soil noninoculated with APR-8 cells							
APR-8 cells applied onto leaves	Leaves	0	0*/$7.0 \times 10^4 \pm 3.2 \times 10^3$	ND	ND	$3.0 \times 10^5 \pm 1.4 \times 10^4$	$6.5 \times 10^5 \pm 4.2 \times 10^4$
Leaves untreated with APR-8 (control)	Leaves	0	0	ND	ND	0	0

Note: CFU values (mean ± standard deviation) were calculated from five replicate experiments.

*In the numerator, the CFU value determined before APR-8 cells were applied onto the leaves; in the denominator, the value determined after the application of APR-8 cells onto the leaves.

of a leaf was excised and placed in a tube containing 5 ml of a sterile 0.5% NaCl solution. The tubes were placed on a shaker for 30 min. Tenfold serial dilutions of the suspension obtained (up to 10^{-3}) were plated (0.1 ml of each dilution) on selective MMA containing kanamycin (50 μg/ml) and streptomycin (500 μg/ml). The number of APR-8 cells was also determined in the soil under the maize. For this purpose, tenfold serial dilutions of soil samples (from 10^{-2} to 10^{-7}) taken from each flask were plated on the selective medium. After five- to seven-day incubation periods, the grown colonies of the APR-8 strain were counted. The number of colony-forming units (CFU) was calculated per 1 cm^2 of the leaf surface.

Plant colonization by PPFMB directly applied onto the leaves. Both maize and APR-8 strain were grown as described in the preceding experiment (see the variant in which APR-8 cells were not introduced into the soil). When the plant height reached nine to ten cm (seven to eight day of the experiment), the upper part of a four to five-cm leaf was plunged into a bacterial suspension of strain APR-8 (10^{-9} cells/ml) for two min. The third (control) leaf of each plant examined was plunged into an equivalent volume of 0.5% NaCl containing no APR-8 cells. Colonization of leaves with APR-8 cells was estimated as the number of CFU/cm^2 of the leaf surface as described for the foregoing experiment.

Analysis of the *Methylobacterium* antagonistic properties. Antagonistic properties of various *Methylobacterium* strains were studied using the perpendicular streak test. The following phytopathogenic bacteria provided from the Department of Phytopathogenic Microorganisms of the Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, served as the test cultures: *Xanthomonas campestris* pv. *campestris* 8003b, *Pseudomonas syringae* pv. *syringae* 8511, *Erwinia carotovora* subsp. *carotovora* 8982, *Clavibacter michiganense* subsp. *michiganense* 13a, and *Agrobacterium tumifaciens* 8628.

The *Methylobacterium* strain was inoculated as a streak along the diameter of a petri dish (50 mm in diameter) containing 20 ml GPA. After incubation for three days, three to four strains of the phytopathogenic bacteria (10^{-9} cells/ml) were applied as streaks at right angle to the *Methylobacterium* streak. The zones of growth inhibition were measured two to three days after coincubation of PPFMB and phytopathogenic bacteria.

Study of ice nucleation in the presence of PPFMB. The effect of PPFMB on ice nucleation was studied in a Selena chamber in the Ukrainian Research

Table 3. Antagonistic properties of the microbial associations including PPFMB

Designations of the bacterial strains or microbial associations	Size of growth-inhibition zones of test cultures (mm)				
	Xanthomonas campestris pv. *campestris* 8003b	*Pseudomonas syringae* pv. *syringae* 8511	*Erwinia carotovora* subsp. *carotovora* 8982	*Clavibacter michiganense* subsp. *michiganense*	*Agrobacterium tumifaciens* 8628
B12*	21 ± 5	26 ± 6	20 ± 8	40 ± 3	19 ± 2
B121	0	0	0	0	0
B122	0	0	0	0	0
B121 + B122	24 ± 2	32 ± 5	15 ± 2	40 ± 3	20 ± 6
Ch8	0	0	0	0	0
Ch8 + B122	18 ± 3	25 ± 4	15 ± 2	30 ± 4	18 ± 3
D10	0	0	0	0	0
D10 + B122	10 ± 3	9 ± 2	6 ± 3	16 ± 4	4 ± 1
LKL	0	0	0	0	0
LKL + B122	17 ± 4	25 ± 5	11 ± 3	40 ± 4	15 ± 3
APR-8	0	0	0	0	0
APR-8 + B122	18 ± 3	25 ± 4	16 ± 3	40 ± 2	19 ± 4
K2	0	0	0	0	0
K2 + B122	11 ± 2	25 ± 3	10 ± 2	27 ± 3	8 ± 2

Note: The size of growth-inhibition zones (mean ± standard deviation) was calculated from three replicate experiments.

*B12 is a mixed culture of epiphytic bacteria from which *M. extorquens* and epiphytic bacteria unable to grow on methanol were isolated (B121 and B122, respectively).

Hydrometeorological Institute using the technique described by Kiprianova *et al.* [8]. A 2-day culture of PPFMB grown on GPA was used to prepare bacterial suspensions in bidistilled water (10^8 cells/ml). Fifty 25-μl drops of a bacterial suspension and two 25-μl drops of bidistilled water (control) were applied with a syringe onto a copper plate. The plate was placed in a Selena chamber, where the temperature decreased from –1 to –15°C. Visual observation and counting of the frozen drops was conducted through a glass cover of a chamber.

RESULTS AND DISCUSSION

Maize colonization with methylotrophic bacteria. In the model experiments, we studied possible ways of maize leaf colonization by methylotrophic bacteria. To ensure selective conditions for revealing the methylotrophic bacteria, which were either inoculated into soil or applied onto leaves, we used a marked strain resistant to antibiotics. This made possible the use of a selective medium supplemented with a high concentration of antibiotics to inhibit the resident methylotrophic bacteria in the samples studied. The marked strain was generated by transferring the conjugative plasmid pULB113, which encodes resistance to antibiotics, from the donor cells of *Methylomonas rubra* 15sh (pULB113) into the recipient cells of *Methylobacterium mesophilicum* 4181 (Str^R). The transconjugant clones *M. mesophilicum* 4181 (Str^R, pULB113) were purified from the donor cells by several repeated platings of individual colonies onto MMA containing kanamycin (50 μg/ml) and streptomycin (500 μg/ml). Under these conditions, the parental stains did not grow: the strain *Methylomonas rubra* 15sh (pULB113) is unable to grow in the presence of streptomycin, whereas *Methylobacterium mesophilicum* 4181 (Str^R) does not grow in the presence of kanamycin. The selected transconjugants were also resistant to ampicillin (300 μg/ml) and tetracycline (40 μg/ml). Thus, we obtained a number of clones of *M. mesophilicum* 4181 (Str^R) which had acquired three various resistances conferred by plasmid pULB113. Like the original strain, transconjugants *M. mesophilicum* 4181 (Str^R) were resistant to streptomycin (500 μg/ml).

One of the transconjugants selected, *M. mesophilicum* APR-8 (Str^R, pULB113), served as a marker strain to study the ways of maize colonization. In our further experiments, selective MMA medium containing kana-

Table 4. Effect of bacteria on ice nucleation

Species, strain		Precent of drops of the bacterial suspension crystallized at a temperature of °C													
		−1	−2	−3	−4	−5	−6	−7	−8	−9	−10	−11	−12	−13	−14
The strains of the genus *Methylobacterium* studied															
M. extorquens	B12						4	6	6	6	6	8	8	10	12
	K2								2	8	12	14	14	18	22
M. mesophilicum	Ch8											2	4	4	8
	D10									2	2	2	4	4	10
	APR-8												2	4	6
	8–18												4	6	8
M. fujisawanse	B5							2	2	6	6	8	12	14	18
	B15						4	4	4	4	6	6	8	10	10
Methylobacterium sp.	T2											4	4	6	8
Strains of the genus *Pseudomonas known to have antifreeze properties**															
P. fragi	4002												2	2	4
P. mendosina	4014												2	4	6
Strains of the genus *Pseudomonas*, active ice nucleators*															
P. fluorescens	19	100													
P. syringae	1947	6	24	40	76	100									
Bidistilled water (control)													10	10	12

* The ice-nucleating capacity of the members of the genus *Pseudomonas* that was reported by Kiprianova *et al*. [8] is given for comparison.

mycin (50 μg/ml) and streptomycin (500 μg/ml) was used to reveal strain APR-8.

Since PPFMB had been previously found not only on plant leaves but also in most soils studied [4, 9], we tried to determine whether plant colonization by methylotrophic bacteria can occur directly from soil (during seed germination). In our model experiments, the cells of the marked strain APR-8 were inoculated into soil where two kinds of seeds were introduced: those pre incubated for 30 min in APR-8 bacterial suspension (10^8 cells/ml) and untreated seeds (Table 2). The control soil contained no APR-8 cells. Both soil and leaves were sampled for 30 days to determine the number of APR-8 cells by plating onto MMA. The results summarized in Table 2 demonstrate that no APR-8 cells were revealed on the maize leaves during these experiments. Hence, no colonization of maize leaves by strain APR-8 occurred directly from soil during seed germination.

In another model experiment, a suspension of the APR-8 cells was applied onto the upper part of two leaves on the day 8 of maize growth (no APR-8 strain was inoculated into soil). Since the maize leaves elongate due to the growth of the basal part of the leaf plate rather than due to apical growth, the APR-8 cells were on the upper part of the leaf during the entire experiment. We determined the number of APR-8 cells on leaves just after their were application and at the end of the experiment (Table 2). The number of APR-8 cells on leaves increased during the vegetation period of maize growth. This indicates that a steady leaf colonization with APR-8 cells occurred. On the control leaf of the same plant, no APR-8 cells were revealed (Table 2). On new leaves that appeared on maize after the beginning of the experiment (on days 12 to 16), we also failed to detect APR-8 cells.

Thus, plant colonization by PPFMB is initiated when these bacteria get onto the plant leaves. When the bacteria are introduced into soil they fail to populate plant leaves during seed germination.

The question arises of whether or not the PPFMB fulfil some functions beneficial to plants. The growing leaves are known to contain methanol of endogenous origin. Since PPFMB assimilate methanol, they successfully compete with other epiphytic bacteria that require polycarbon compounds [1, 3]. In addition, there are data indicating that PPFMB have a favorable effect on plant growth because they synthesize some biologically active compounds, such as cytokinins, PQQ, urease, and polyhydrooxybutyrate [10–13]. We suggested that PPFMB can fulfil some protective function for plants. So we studied whether they can inhibit the

growth of phytopathogenic bacteria or have an effect on ice nucleation.

Antagonistic properties of PPFMB. Table 3 demonstrates that only mixed cultures of epiphytic bacteria including PPFMB (e.g. B12) suppressed the growth of phytopathogenic bacteria, whereas none of the pure PPFMB cultures studied (B121, Ch8, D10, LKL, APR-8, K2) exhibited this effect. We isolated monocultures from the mixed cultures to determine the agents that are responsible for the antagonistic properties of the mixed cultures. Thus, *M. extorquens* B121 [4] was isolated from the mixed culture B12, as well as the unidentified epiphytic bacterium B122, which cannot utilize methanol. Each of these monocultures produced no antagonistic effect on phytopathogenic bacteria (Table 3). A reconstructed artificial microbial association containing strains B121 and B122 at a ratio of 1 : 1 inhibited the growth of phytopathogenic bacteria with an efficiency similar to that of the natural mixed culture B12 (Table 3). We also constructed the artificial microbial associations containing one of the collection *Methylobacterium* strains and strain B122. It turned out that almost all of these microbial associations inhibited the growth of phytopathogenic bacteria (Table 3).

The above evidence indicates that PPFMB are involved in plant protection against phytopathogenic bacteria when they are the components of mixed epiphytic cultures.

PPFMB involvement in ice nucleation. Strains of epiphytic bacteria are known which catalyze ice nucleation from bidistilled water at temperatures of –2 to –5°C, whereas ice nucleation in bidistilled water usually occurs at –20°C [14, 15]. Serving as a bacterial ice nucleus, some strains of phytopathogenic bacteria (e.g. *Pseudomonas syringae* and *Erwinia herbicola*) promote frost injury to plant tissues, which results in plant infection and finally in plant death [14, 15]. Thus, we studied whether the epiphytic PPFMB can promote ice nucleation.

The results summarized in Table 4 demonstrate that cells of different PPFMB strains differed in their capacity to serve as ice nucleus. Thus, in the presence of some *Methylobacterium* strains, ice nucleation was initiated at temperatures from –6 to –8°C; other strains promoted ice nucleation at a temperature lower than –12°C. In the absence of bacterial cells (control), ice nucleation was initiated at –12°C. Hence, PPFMB on plant leaves do not catalyze water freezing and, moreover, some PPFMB strains have an antifreeze effect. Thus, our results indicate that the pink-pigmented facultative methylotrophic bacteria which inhabit plant phyllosphere do not catalyze ice nucleation and do not increase frost injury to plants. It might be that some strains of epiphytic PPFMB exhibit an antifreeze effect under natural conditions.

Thus, we have demonstrated in the model experiments that PPFMB applied onto maize leaves can colonize them. However, no colonization of maize leaves occurred during seed germination when PPFMB were introduced into soil. Our results suggest that PPFMB inhabiting plant phyllosphere enter soil with dead leaves in autumn, whereas in spring, during vegetative plant growth, they recolonize the plant leaves being transferred to their surface by air with soil particles. Monocultures of PPFMB produced no antagonistic effect on phytopathogenic bacteria. However, mixed bacterial cultures containing *M. mesophilicum* or *M. extorquens* were antagonistic to the phytopathogenic bacteria *Xanthomonas campestris*, *Pseudomonas syringae*, *Erwinia carotovora*, *Clavibacter michiganense*, and *Agrobacterium tumifaciens.* Unlike several other species of epiphytic bacteria, the strains of *Methylobacterium extorquens*, *M. mesophilicum* and *M. fujisawaense*, do not catalyze ice nucleation. Hence, they do not increase frost injury to plants. Our results indicate that the coexistence of PPFMB and plants is mutually beneficial and the bacteria can be protective for plants.

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