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EXPERIMENTAL ARTICLES

Facultative and Obligate Aerobic Methylobacteria Synthesize Cytokinins

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Abstract—The presence and expression of genes controlling the synthesis and secretion of cytokinins by the pink-pigmented facultative methylotroph *Methylobacterium mesophilicum* VKM B-2143 with the serine pathway and nonpigmented obligate methylotroph *Methylovorus mays* VKM B-2221 with the ribulose monophosphate pathway of C_1 metabolism were shown using the polymerase chain reaction (PCR) and reverse transcription–PCR methods. The presence of the corresponding mRNA in *M. mesophilicum* cells grown on methanol or succinate suggests that the expression of these genes is constitutive. The cytokinin activity of culture liquid and its fractions was determined by a biotest with *Amarantus caudatus* L. seedlings. Using enzyme-linked immunosorbent analysis, we detected zeatin (riboside) in the culture liquid of both bacteria studied. The data obtained show that the aerobic methylobacteria are phytosymbionts that are able to utilize the single- and polycarbon compounds secreted by symbiotic plants and to synthesize cytokinins.

Key words: aerobic methylotrophic bacteria, *Methylobacterium mesophilicum*, *Methylovorus mays*, cytokinins, zeatin (riboside), *tmr*, *tzs*, and *ptz* genes

Since the discovery of cytokinins, it has been assumed that these phytohormones are produced by plants. Recently, however, Holland has hypothesized that cytokinins are synthesized by phytosymbiotic microorganisms, methylotrophic bacteria in particular, rather than by plants themselves [1]. This original hypothesis explains the reasons for intimate symbiosis between methylotrophs and higher plants, although it needs a strong experimental basis.

Aerobic methylotrophic bacteria (methylobacteria) abundantly colonize leaf surfaces and are encountered in the seeds and rhizosphere of many plants [2–5]. This is often explained by the functioning of the methanol cycle, i.e., the formation and secretion of methanol by plants (estimations show that as many as 100 million tons of methanol are annually synthesized by plants) and the active utilization of this methanol by phytosymbiotic aerobic methylobacteria as the source of carbon and energy [6]. The relationship between plants and associated methylobacteria is obviously not limited only to commensalism, since the phytohormones synthesized by plants were found to be necessary for phytopathogenic and phytosymbiotic microorganisms [7], and aerobic methylobacteria were found to be able to synthesize cytokinins necessary for plants. Using the polymerase chain reaction (PCR) method, Long *et al.* [8] revealed the adenylate isopentenyltransferase (cytokinin synthase) gene in the facultative methylotroph *Methylobacterium extorquens.* We also found that the genome of various methylotrophs contains nucleotide sequences homologous to the genes controlling the synthesis and secretion of cytokinins [5]. This prompted us to study the metabolic interaction of aerobic methylobacteria with symbiotic plants in more depth.

The aim of the present work was to study the ability of two species of obligate and facultative aerobic methylobacteria to synthesize cytokinins.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. Bacteria used in this study were the pink-pigmented facultative methylotroph *Methylobacterium mesophilicum* VKM B-2143 (=JCM 2829 = DSM 1708) and the nonpigmented obligate methylotroph *Methylovorus mays* VKM B-2221. The bacteria were grown in K medium with 0.5% methanol [9]. Cells were harvested by centrifugation at 10000 *g* for 30 min in the late logarithmic phase (70 and 30 h of growth for *M. mesophilicum* and *M. mays*, respectively). The supernatant of the culture liquid was lyophilized and stored to be later used for the analysis of cytokinins.

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DNA isolation. Wet biomass (about 20 mg) was suspended in 0.5 ml of TE buffer (10 mM Tris–HCl + 1 mM EDTA, pH 7.6). The suspension was supplemented with 0.5% sodium dodecyl sulfate (SDS), added in the form of 10% solution, and with 100 μg/ml proteinase K (Promega). After 1-h incubation at 37°C, the suspension was supplemented with 0.7 M NaCl and 1% cetyltrimethylammonium bromide (CTMAB), added as 10% solution, and incubated for 10 min at 65°C. The suspension was then sequentially extracted with equal volumes of a chloroform and a phenol–chloroform (1 : 1) mixture. DNA from the aqueous phase was precipitated with 0.6 volume of isopropanol, washed with 70% ethanol, precipitated by centrifugation at 10000 *g* for 10 min, and dissolved in 100 μl of H_2O.

PCR amplifications were carried out in a reaction mixture (50 μl) containing 10 ng DNA, 250 μM each of deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 30 μM of primer, 50 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml BSA, 0.02% NP-40, and 2 U Taq DNA polymerase. After preliminary denaturation at 95°C for 5 min, 30 standard three-step PCR cycles were performed: DNA denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and DNA synthesis at 72°C for 1 min. The DNA from *Agrobacterium tumefaciens* was used as the positive control. PCR products were analyzed by electrophoresis in 2% agarose gel.

RNA isolation. The wet biomass (about 20 mg) was washed with TE buffer and suspended in 0.2 ml of lysing buffer (4 M guanidine isothiocyanate, 0.2 M mercaptoethanol, and 2% *N*-lauroylsarcosine). Tubes with this suspension were incubated and occasionally shaken until complete cell lysis occurred. The lysate was then mixed with an equal volume of phenol (pH 4.0; 60°C), 0.5 volume of 0.1 M sodium acetate (pH 5.2), and an equal volume of a chloroform–isoamyl alcohol (24 : 1) mixture. The tubes were vigorously shaken for 10 min, rapidly cooled on ice, and centrifuged at 2000 *g* for 10 min at 4°C. The aqueous phase was collected and reextracted once with a phenol–chloroform mixture and twice with chloroform. After the subsequent addition of 2 volumes of ethanol, the mixture was kept at –20°C for 2 h and RNA was then precipitated by centrifugation at 1200 *g* for 20 min at 4°C. The precipitate was dissolved in 20 μl of 40 mM Tris–HCl buffer (pH 7.9) containing 10 mM NaCl and 6 mM $MgCl_2$. The mixture was supplemented with 40 U of the ribonuclease inhibitor RNasin (Promega) and 1 U of RNase-free DNase (Promega) and incubated at 37°C for 20 min. DNase was then inactivated by heating at 70°C for 10 min. The resulting RNA preparation was cooled and stored to be used later for the synthesis of the first strand of cDNA.

Synthesis of the first strand of cDNA was carried out in a reaction mixture (50 μl) containing 22 μl of the above RNA preparation, 20 pM primer, 50 mM Tris–HCl (pH 8.3), 40 mM KCl, 7 mM $MgCl_2$, 100 μg/ml BSA, and 250 μM each of deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, and dTTP). After 5-min preincubation at 65°C, the mixture was cooled for 15 min to anneal the primer and then supplemented with 10 U of RNasin and 23 U of AMV reverse transcriptase (Promega). The mixture was incubated at 42°C for 1 h, after which it was heated at 90°C for 3 min and then rapidly cooled on ice. Two μl of this mixture was used to perform the PCR amplifications as described above.

The oligonucleotides used in this work were as follows:

(1) 5'-CTTGATCGTGTGCAATGCTGT-3'
(2) 5'-ATTGAGAAGCGAAATCGACCC-3'
(3) 5'-TGCGCCATCTTCTGGCCACTG-3'
(4) 5'-ATCCTGAAGAGCTACAATCAAC-3'
(5) 5'-AGTTTAGCATTAAGGTCTGACTGA-3'

Extraction and analysis of phytohormones. The lyophilized culture liquid was suspended in 80% methanol and kept at 4°C for 16 h, after which the methanol was evaporated and the aqueous residue was extracted with water-saturated butanol at pH 7.5 and with ethylacetate at pH 2.5–2.8. The ethylacetate and butanol extracts were dried and dissolved in 80% ethanol [10].

Cytokinins present in these preparations were fractionated by thin-layer chromatography (TLC) on F-254 Silufol plates (Czech Republic) developed in an isopropanol–benzene–ammonia (4 : 1 : 1) solvent system. Spots fluorescent under UV light were eluted with 80% ethanol. Cytokinins were identified using the authentic samples of zeatin, zeatin riboside, benzylaminopurine, isopentenyladenine and isopentenyladenosine purchased from Sigma.

UV spectra were recorded on a Shimadzu-UV-160 spectrophotometer (Japan).

Cytokinins were analyzed by the ELISA method. After the interaction of adsorbed and free antigen with rabbit antibodies against the respective phytohormone, the reaction products were detected using the antirabbit peroxidase-labeled conjugant. The reagents necessary for this analysis were purchased from AO Uralinvest (Ufa, Russia) [11].

Biotesting of cytokinin activity in the culture liquid and the TLC fractions was performed using *Amarantus caudatus* seedlings [12]. For this purpose, the ethanol eluates of TLC spots were placed in 4-cm-diameter petri dishes and dried. Then, pieces of filter paper soaked with 1.5 ml of 0.02 M Na-phosphate buffer (pH 6.3) containing 1 mg/ml L-tyrosine were placed in these petri dishes (experimental variants) or in pure petri dishes (control variants). The culture liquid (pH 6.3) which was to be tested for cytokinin activity was supplemented with 1 mg/ml L-tyrosine and used, in 1.5-ml aliquots, to soak the filter paper, which was then placed in pure petri dishes. In this case, the control petri dishes contained filter paper soaked with a mineral medium used for the growth of methylobacteria.

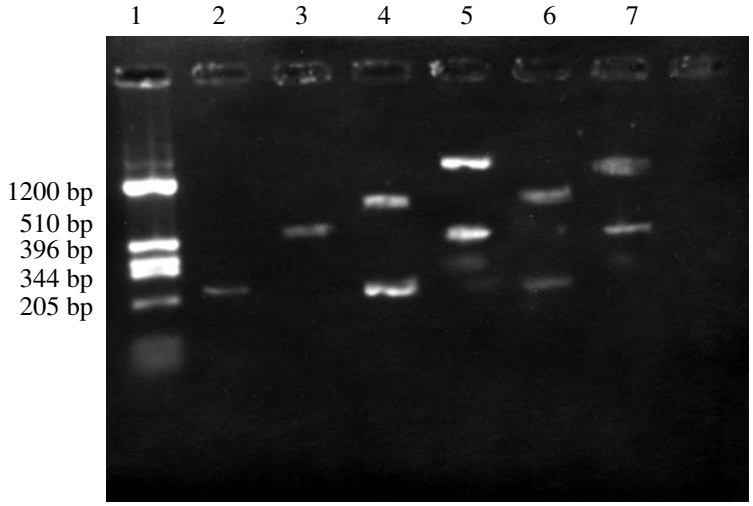

Fig. 1. Electrophoresis of the PCR amplification products of bacterial DNA. Lanes: (1) DNA markers (pTZ19R DNA *Hin*fI digest); (2, 4, and 6) PCR with the primer pair 1–2 of the DNAs of *A. tumefaciens*, *M. mesophilicum*, and *M. mays*, respectively; (3, 5, and 7) PCR with the primer pair 1–3 of the same DNAs.

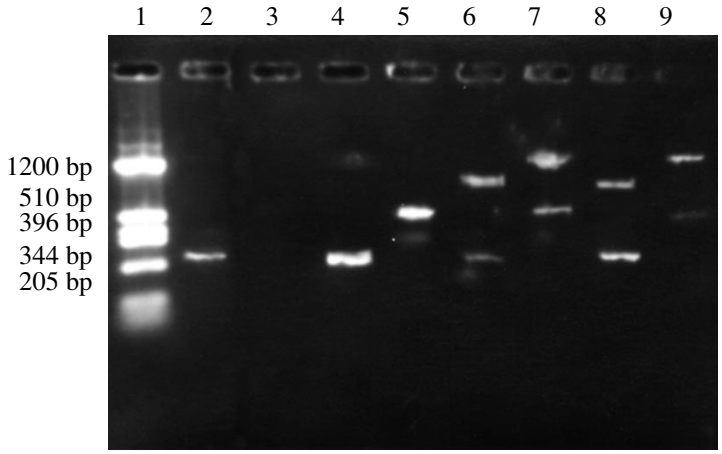

Fig. 2. Electrophoresis of the PCR amplification products of cDNA synthesized on the mRNA of (2–5) *A. tumefaciens* and (6–9) *M. mesophilicum.* Lanes: (1) DNA markers (pTZ19R DNA *Hin*fI digest); (2 and 6) PCR with the primer pair 1–2 of cDNA synthesized on mRNA with primer 2; (4 and 8) PCR with the primer pair 1–2 of cDNA synthesized on mRNA with primer 3; (3 and 7) PCR with the primer pair 1–3 of cDNA synthesized on mRNA with primer 2; (5 and 9) PCR with the primer pair 1–3 of cDNA synthesized on mRNA with primer 3.

A. caudatus seeds were germinated at 24°C for 72 h, after which radicles were cut from the seedlings under illumination with scattered light. The cotyledons were placed in petri dishes prepared as described above (10 cotyledons per each dish), incubated in the dark at 24°C for 18 h, and then transferred to penicillin vials (10 cotyledons from particular dishes per vial) containing 2 ml of water. The cotyledons in the vials were subjected to three freeze–thaw cycles to release betacyanins from the cells. The concentration of betacyanins was determined spectrophotometrically by the difference between the optical densities measured at 542 and 620 nm. The cytokinin activity of the control variants was taken to be 100%.

The data obtained were statistically processed using the Student's *t*-test with a confidence level of 0.95.

RESULTS AND DISCUSSION

Phytohormones are known to play an important role in the interaction of plants with associated microorganisms, both phytopathogenic and symbiotic. In particular, the production of cytokinins is one of the essential factors of virulence for the majority of phytopathogenic bacteria. Methylotrophs living on leaf surfaces were also found to be able to produce cytokinins [8]. In attempts to discern which elements of the methylotroph genome are responsible for cytokinin production, we analyzed the nucleotide sequences of the genes (and the

respective amino acid sequences of their products) that are known to code for the key enzymes of cytokinin synthesis in some phytopathogenic bacteria, such as the *tmr* and *tzs* genes of *Agrobacterium tumefaciens* [13] and the *ptz* gene of *Pseudomonas syringae* pv. *savastanoi* [14]. The comparison of the nucleotide sequences of genes and the predicted amino acid sequences of their products showed a high degree of homology between the 5'-terminal sequences of these genes and the N-terminal amino acid sequences of their products. The C-terminal regions of proteins and the corresponding regions of DNA were found to be variable, although amino acid residues at positions from 25 to 38 and from 92 to 104 turned out to be conservative, and the sequences 31–38 and 96–104 were identical [13]. Genes involved in the biosynthesis of cytokinins in methylotrophs were studied by the PCR method using synthetic oligonucleotide primers homologous to various regions of the *tmr, tzs*, and *ptz* genes.

The oligonucleotide primer 1 codes for amino acids 31–38 of the *tzs* gene product, while the nucleotide sequence of primer 2 is complementary to the sequence encoding amino acids 98–104 of the same protein. Ptz, Tzs, and Ipt proteins encoded by the *ptz*, *tzs*, and *tmr* genes are characterized by 100% homology. The oligonucleotide primer 3 is complementary to the sequence coding for amino acids 224–230 of the *tzs* gene product (this sequence has no homologous regions in the *tmr* and *ptz* genes). Primer 4 corresponds to the sequence coding for amino acids 48–55 of the *ptz* gene product (this sequence has slightly homologous regions in the *tmr* and *tzs* genes). Finally, the oligonucleotide primer 5 is complementary to the sequence coding for amino acids 159–167 of the *ptz* gene product (this sequence has no homologous regions in the *tmr* and *tzs* genes). PCR amplifications were carried out using the genomic DNA of the methylotrophs studied and the following primer pairs: 1–2, 1–3, 1–5, 4–2, 4–3, and 4–5.

The electrophoresis of PCR products showed the presence of distinct bands only when the primer pairs 1–2 and 1–3 were used. With these primer pairs, the DNA of *A. tumefaciens* was used as the positive control. Amplified DNA fragments had the expected sizes (about 220 and 600 bp for the primer pairs 1–2 and 1–3, respectively). However, the electrophoretograms also had bands corresponding to DNA fragments with sizes about twice as large as the expected values (Fig. 1). After separation in agarose gel, all of these DNA fragments were reamplified with the same primer pairs. The electrophoretogram of the PCR amplification products of the 220-bp oligonucleotide with the 1–2 primer pair had one band corresponding to a DNA fragment of the same size, i.e., 220 bp. Similarly, the electrophoretogram of the PCR amplification products of the 600-bp oligonucleotide with the 1–3 primer pair had one 600-bp band. The electrophoretograms of the PCR amplification products of the double-length oligonucleotides with the corresponding primer pairs had two bands with sizes identical to those observed after the first PCR. These data suggest that the genes studied are represented by at least two copies of the gene located close to each other in the genome. Such a doubling of genes is often encountered in microorganisms and probably serves to enhance the gene function. Thus, PCR analysis showed that the genomes of the methylobacteria under study possess nucleotide sequences homologous to the cytokinin synthesis genes of *A. tumefaciens.*

It should be noted that these data provide no indication of the expression of the cytokinin synthase genes in methylobacteria. However, the presence of the corresponding mRNA molecules provides grounds to believe that the given DNA sequence is part of the functionally active gene encoding some enzyme involved in cytokinin synthesis. Using primer 2 or 3, AMV reverse

Table 1. Analysis of the exometabolites of methylobacteria separated by TLC

Substance	R_f	Cytokinin activity, % of the control	λ_{max}, nm
Methylovorus mays			
1	0.15	126	254
2	0.20	138	270
3	0.34	203	256
4	0.41	190	259
5*	0.55	226	263
6	0.86	172	271
7	0.92	147	278
8	0.96	149	271
Methylobacterium mesophilicum			
1	0.15	162	265
2	0.25	122	252
3	0.36	154	241
4	0.41	158	263
5*	0.50	348	262
6	0.63	219	264
7	0.96	149	229

* According to their R_f values and spectral maxima, substance 5 corresponds to zeatin riboside.

Table 2. ELISA of substances 5 (see Table 1), presumably representing zeatin riboside

Methylobacterium	Estimated amount of zeatin riboside, pg-eq./0.1 ml	
	without dilution	after 10-fold dilution
M. mesophilicum	50	5
M. mays	1000	100

transcriptase, and RNA isolated from *M. mesophilicum*, and *M. mays* cells grown either methylotrophically (on K medium with methanol) or heterotrophically (10 passages on K medium with 0.3% succinate), we synthesized and used the respective cDNAs for PCR amplifications with the primer pairs 1–2 and 1–3. In these experiments, the RNA of *A. tumefaciens* was used as the positive control.

When the primer pair 1–2 was used, the PCR of the cDNA synthesized on *A. tumefaciens* mRNA led to the amplification of DNA fragments of the expected size (about 220 bp). When the primer pair 1–3 was used, the PCR led to the amplification of 600-bp DNA fragments only on cDNA synthesized with primer 3 (Fig. 2). These results agree well with the known structure of the *tzs* gene of *A. tumefaciens*. However, when the mRNAs from *M. mesophilicum* (Fig. 2) and *M. mays* (data not shown) were used, the PCR led to the amplification of two DNA fragments about 220 and 800 bp in size in the PCR with primers 1–2 and about 600 and 1200 bp in size in the PCR with primers 1–3. These data can again be interpreted to indicate the presence of two copies of the same gene located close to each other in the genome of methylotrophic bacteria and their transcription on the same mRNA. The fact that the respective mRNA occurs in *M. mesophilicum* cells grown on either 0.3% methanol or 0.3% succinate indicates that the expression of the PHB synthase gene is constitutive or at least that methanol does not induce the expression. Thus, *M. mesophilicum* and *M. mays* cells contain the mRNA necessary for the synthesis of proteins responsible for cytokinin production. This implies that the corresponding genes of cytokinin synthesis are functionally active.

The chromatographic analysis of the culture liquids and biomasses of methylobacteria showed that their cytokinins occur primarily in the culture liquid; i.e., they are exometabolites. The cytokinin activities of the culture liquids of *M. mesophilicum* and *M. mays* determined in biotests with *A. caudatus* seedlings were 273 and 263%, respectively.

Table 1 summarizes the R_f values (TLC) of substances isolated from the culture liquid of the methylobacteria, as well as their cytokinin activities and spectral maxima. It can be seen that most of these substances possess cytokinin activity and have spectral maxima at wavelengths typical of cytokinins. The different chromatographic mobilities of these substances can be explained by the ability of cytokinins to form complexes with other cellular constituents, such as nucleotides, ribose, and polysaccharides [15]. The maximum cytokinin activity was revealed in sample 5 of both of the methylobacteria studied, which contained substances corresponding to zeatin riboside in the R_f value and spectral maximum. The analysis of these samples by ELISA (Table 2) showed that the culture liquid of methylobacteria contains zeatin and/or zeatin riboside. The proportional decrease in cytokinin activity of samples observed with a 10-fold dilution implies that the immunoreaction was specific.

In conclusion, the pink-pigmented facultative methylotroph *M. mesophilicum*, and the nonpigmented obligate methylotroph *M. mays*, produce the extracellular phytohormones cytokinins. *M. mesophilicum* was isolated from the phyllosphere of the rye grass *Lolium perenne* L. [16] and *M. mays* was isolated from the phyllosphere of the maize *Zea mays* L. [17]. The data obtained here show that these methylobacteria are not fortuitous inhabitants of leaf surfaces but are instead phytosymbionts utilizing the C_1 substrates secreted by symbiotic plants and providing them with phytohormones. In the facultative methylotroph *M. mesophilicum*, the genes involved in cytokinin synthesis are expressed in the cells grown both methylotrophically and heterotrophically. Therefore, the methylotrophs under study synthesize cytokinins constitutively.

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