Microbiology, Vol. 70, No. 6, 2001, pp. 623–632. Translated from Mikrobiologiya, Vol. 70, No. 6, 2001, pp. 725–736.

REVIEW PAPERS

Aerobic Methylotrophic Bacteria as Phytosymbionts

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Received April 1, 2001; in final form, April 28, 2001

Abstract—This paper deals with the physiological, biochemical, and molecular genetic aspects of the interaction of aerobic methylotrophic bacteria with plants by means of phytohormones (such as cytokinins and auxins) and other physiologically active substances (vitamins, exopolysaccharides, bioprotectants, and others). An overview of the field and the prospects of research in the field of bacteria–plant interactions and the application of aerobic methylotrophs in plant biotechnology is discussed.

Key words: aerobic methylotrophic bacteria, phytosymbiosis, cytokinins, auxins.

Aerobic methylotrophic bacteria utilize methane (methanotrophs) or its oxidized and substituted derivatives (methylobacteria) as sources of carbon and energy. Two major groups of methylotrophs can be distinguished, depending on the range of utilizable substrates. Obligate methylotrophs can utilize only one-carbon compounds, whereas facultative methylotrophs utilize, in addition, various multicarbon compounds. There are three primary pathways of C_1-metabolism: the serine, the ribulose monophosphate, and the ribulose bisphosphate pathways [1].

Until recently, the primary role of aerobic methylotrophs was considered to be their involvement in the global cycling of carbon, particularly in the oxidation of methane, whose greenhouse effect is 20 times more severe than that of carbon dioxide. The increased concentration of methane in the atmosphere is due to both abiotic (geochemical) and biotic (methanogenic) sources. Conversely, aerobic methane-oxidizing communities (the so-called bacterial gas filters), which function in many ecosystems [2, 3], reduce the amount of methane entering the atmosphere. Methylobacteria can mineralize halogenated methane derivatives [4], methylated sulfur-containing compounds [5, 6], and methylated amines [7].

In recent years, researchers have discovered another important function of methylotrophs. The point is that methanol and other C_1-compounds are natural products of plant metabolism, due to which methylotrophs, which utilize these compounds, are ubiquitous in the plant phyllosphere and rhizosphere, where they efficiently compete with other microorganisms. In this case, methylotrophic bacteria not merely colonize plants but are symbiotically related to them. This paper is an attempt to review recent data on the symbiotic relationship between aerobic methylotrophic bacteria and plants.

DISTRIBUTION OF AEROBIC METHYLOTROPHIC BACTERIA

Aerobic methylotrophic bacteria are ubiquitous in the phyllosphere and rhizosphere of plants and often colonize their seeds.

The most abundant methylotroph, which is found in the phyllosphere of more than 50 plant species, is a typical pink-pigmented facultative methylobacterium (PPFM), *Pseudomonas extorquens* [8]. This bacterium was first isolated from the liverwort *Scapania nemorosa* and was suggested to be responsible for the growth stimulation of this plant under laboratory conditions [9]. Another pink bacterium, *Pseudomonas mesophilica*, was isolated from the rye grass *Lolium perene* [10, 11]. This bacterium was found to be similar to the other pink-pigmented facultative methylotrophs known at the time [9, 12, 13] and was reclassified into *Methylobacterium mesophilicum* [14]. PPFM add up to 79% of the total number of the heterotrophs found in the phyllosphere [15]. *M. mesophilicum* was found on leaves of more than 40 plant species in amounts from 0.5 to 69.4 CFU/cm^2, even if the leaves were preliminarily surface-sterilized [16].

Romanovskaya *et al.* detected PPFM in the phyllosphere of more than 200 medicinal, decorative, agricultural, and wild plants in Ukraine [17]. Pink methylotrophs are also present in soil. Some researchers assume that PPFM colonize the phyllosphere in spring, borne by the soil dust. This assumption is confirmed by the fact that the plant leaves that are well off the soil surface are much less colonized by PPFM than those situated near the soil. The phyllosphere methylobacteria are highly resistant to dehydration, freezing on hygroscopic carriers, UV and ionizing radiation, and elevated temperatures. PPFM may remain viable after UV irradiation in the doses that are lethal to enterococci, pseudomonads, and methanotrophs [18].

0026-2617/01/7006-0623$25.00

Recently, we have isolated not only PPFM but also yellow pleiomorphic bacteria of the genus *Xanthobacter*, coccoid nonpigmented bacteria of the genus *Paracoccus* [19], as well as obligate and restricted facultative methylobacteria of the genera *Methylobacillus*, *Methylophilus*, and *Methylovorus* [20] from the phyllosphere and rhizosphere of 140 plants growing in the near-Moscow region. These bacteria formed up to 90 colonies per cm^2 of the leaf surface. They were also found as components of the rhizosphere-, seed-, and fruit-living endophytic microcommunities. When methylobacteria were isolated from the living needles of conifers in winter, their growth on selective agar media was characterized by a two-week lag phase, which was not observed if the isolation was carried out in summer. It is possible that, in winter, methylobacteria occur in the intercellular space or under the cuticle of leaves rather than on their surface.

It was shown that methanotrophs can form symbiotic associations with various aquatic plants [21, 22] and live not only in the rhizosphere and rhizoplane of rice [23] but also in the root epidermis and vascular tissues [24]. Of great interest are the psychrophilic methanotrophic communities of sphagnum peatlands in the tundra and permafrost areas. The sphagnum bogs of West Siberia and the northern part of the East European Plain are dominated by acidophilic, either psychrotrophic or mesophilic, methanotrophs [25, 26], one of which has recently been isolated in pure culture and identified as *Methylocella palustris* [27]. The first neutrophilic psychrophilic methanotroph, *Methylobacter psychrophilus*, was isolated from the sphagnum peat of shrubby tundra north of the city of Vorkuta [28].

Further studies showed that a vast area of northern Russia from the Polar Urals in the West to the Chukchi and Kamchatka Peninsulas in the East, which lies in the tundra and permafrost zone, works as a psychrophilic methane-oxidizing plant–bacterial filter capable of controlling the flow of methane from the tundra soil to the atmosphere. In this filter, methanotrophs were found not only on the surface but also in the hyaloplasm of sphagnum mosses and in the vascular tissues of sedges [29, 30]. The PCR analysis of psychrophilic methanotrophic communities in soils of the northern taiga and subarctic tundra showed the presence of methanotrophs of the genera *Methylobacter*, *Methylomonas*, *Methylosphaera*, and *Methylomicrobium* [31].

The close association of aerobic methylobacteria with plants is now explained by the functioning of the so-called methanol cycle, which involves the formation and excretion of methanol by the plants and its utilization by the methylobacteria as a source of carbon and energy. It is known that the atmospheric methanol produced by plants makes up to 100 Tg C, or 40–46% of the total volatile atmospheric organic carbon [32, 33]. The main source of methanol in plants is the demethylation of the cell-wall pectin by pectin methylesterase. Other sources of methanol are intermediates of the tetrahydrofolate pathway, protein methyltransferases, and the degradation of lignin in secondary cell walls. Methanol is produced during the growth of cells and the formation of the intercellular space. The methanol formation rate is higher in young than in mature cells. The methanol produced in plant leaves is evaporated through their stomates and is emitted from the leaf cuticle. When the cuticle is diminished, the amount of the emitted methanol decreases [34]. Scanning electron microscopic studies showed that the microcolonies of methylobacteria grown on the leaf surface are covered by a cuticle or polysaccharides [15]. There is evidence that methylobacteria can also penetrate into the intercellular space. In addition to methanol, higher plants may produce methylated amines [7] and algae may produce methylated sulfuric compounds [5].

EFFECT OF METHYLOTROPHIC BACTERIA ON THE GROWTH AND DEVELOPMENT OF PLANTS IN VIVO

Methylobacteria not merely live on the surface of seeds but play a role in the process of seed germination and in the conservation of seed germination capacity during storage. For instance, the exposure of soybeans to 50°C for 48 h led to a decrease in their germination rate and to a 95–97% fall in the population of symbiotic PPFM. However, six to eight weeks after the heat treatment of the soybeans, the PPFM population was restored. The heat treatment obviously reduced the germination rate of the soybeans due to a decrease in the amount of methylobacteria on their surface, since the reinoculation of the heat-treated soybeans with a PPFM culture or washed PPFM cells augmented the germination rate to values even exceeding the initial one [35]. The seedlings grown from the heat-treated soybeans markedly differed from the control seedlings grown from untreated soybeans: the biomass of the experimental seedlings was 75% lower than that of the control seedlings, due to the poorer growth of roots. At the same time, the seedlings grown from the thermally treated soybeans reinoculated with PPFM showed the good growth and normal development of the root system, so that their biomass exceeded the control seedling biomass. The biomass of the seedlings grown from the untreated soybeans inoculated with the PPFM exceeded the control seedling biomass by 30% [35].

The spraying of the corn [36], spinach, beet, and soybean [37] plants with 20% methanol augmented their yield under laboratory conditions. The field experiments with soybean plants, in which the population of symbiotic PPFM was evaluated 10 days after the spraying of the plants with 20% methanol, showed that the spraying led to a twofold increase in the PPFM population and to a 45% increase in the soybean yield as compared with the control plants. Similar experiments with plants grown from the heat-treated and untreated soybeans in a greenhouse showed that only the control plants, which were grown from the heat-untreated soy-

beans and so were normally populated by the PPFM, responded to the methanol spraying by enhancing the crop yield. Holland explained these observations by the ability of the PPFM to produce the phytohormones cytokinins. Namely, exogenous methanol stimulates the growth of methylotrophs, which provide plants with cytokinins; in turn, the bacterial cytokinins stimulate plant growth, which leads to an additional excretion of metabolic methanol by the plants and thereby stimulates the growth of the methylotrophs. Thus, the methanol cycle becomes closed [35].

EFFECT OF METHYLOTROPHIC BACTERIA ON THE GROWTH AND DEVELOPMENT OF PLANTS IN VITRO

The aerobic nonpigmented obligate methylobacterium *Methylovorus mays* was found to exert a beneficial effect on the growth and morphogenesis of tobacco, potato, and fiber flax grown in vitro [38]. During clonal reproduction, the colonization of tobacco and potato grafts by *M. mays* promoted their growth and root formation, especially when the grafted plants were grown in the absence of vitamins and phytohormones. In the cultivation media without sucrose, the plants colonized by *M. mays* could grow, albeit slowly, whereas the uncolonized plants died within 7–10 days of cultivation. The colonization of the tobacco and flax explants by methylobacteria considerably accelerated their regeneration and the formation of roots in the course of the subsequent cultivation of the regenerants. It should be noted that the number of methylobacteria on the new generations of grafts did not decrease, indicating a close association between the methylobacteria and the plants cultivated in vitro.

The methylobacterial colonization of transgenic tobacco plants expressing the cytokinin *ipt* gene of agrobacteria restored the normal process of root formation. Such transgenic plants are known to produce large amounts of cytokinins, which leads to their dwarf state, bushiness, the absence of roots, and male sterility. This implies that the restoration of the process of root formation in the transgenic plants colonized by methylobacteria may be due to the ability of these bacteria to synthesize not only cytokinins but also other growth regulators capable of normalizing the impaired phytohormonal status of such plants.

METHYLOTROPHIC BACTERIA AND THE NITROGEN METABOLISM OF PLANTS

It is known that the symbiosis between bacteria and plants is largely determined by the ability of these bacteria to fix atmospheric nitrogen. Most methanotrophs are able to fix molecular nitrogen [39]. The methanotrophs of sphagnum peatlands can also fix nitrogen, although they are not primary nitrogen fixers in these habitats [39, 40]. Of great interest are some recent isolates of nitrogen-fixing psychrophilic acidophilic sphagnum-associated methanotrophs that possess antibacterial properties [3].

Unlike many other nitrogen-fixing bacteria, facultative methylobacteria of the genus *Xanthobacter* can fix molecular nitrogen under chemolithoautotrophic conditions [41]. Inhabiting the rhizosphere of rice grown in anoxic paddy soils with a high concentration of H_2, CO_2, and other products of anaerobic metabolism, such as organic acids and alcohols, methylotrophic xanthobacters may considerably contribute to the nutrition of rice plants and nitrogen balance in the rice rhizosphere [42].

PPFM are involved in the nitrogen metabolism of colonized plants [43]. Soybean plants have several urease isoenzymes: the Eu1 urease located in beans, the Eu4 urease located in all plant tissues, and the Eu2 and Eu3 ureases, which are necessary for the normal urease activity of soybean plants. In the soybean plants with the mutant *eu3-e1/eu3-e1* gene, urea was accumulated in the plant tissues because of impaired urease activity. The colonization of such plants by PPFM did not restore their urease activity. At the same time, the colonization of the double *eu1-sun/eu1-sun, eu4/eu4* soybean mutants by PPFM led to the restoration of their urease activity to a level of 20–40% of that of the wild-type plants, due to the PPFM urease.

SYNTHESIS OF PHYTOHORMONES AND OTHER BIOACTIVE SUBSTANCES BY METHYLOTROPHIC BACTERIA

Cytokinins are phytohormones whose molecules represent adenine derivatives with a substituent at the N-6 atom of the purine ring. The nature of the substituent determines the physiological activity of cytokinins [44]. The cytokinins regulate many physiological processes in plants; particularly they stimulate the division of plant cells [45, 46], remove apical domination, activate dormant buds, induce seed germination [47], and retard the senescence of cut leaves. Furthermore, they enhance the resistance of plant cells to various adverse factors: extreme temperatures, drought, fungal and viral infections, mechanical damage, and toxic chemical agents [44]. Cytokinins also regulate the formation of chloroplasts at the early stages of leaf development, inducing the biosynthesis of chlorophyll, nucleic acids, and chloroplast proteins [48]. Bound to a protein receptor, the cytokinins enhance the activity of RNA polymerases and the matrix activity of chromatin, thereby increasing the number of polyribosomes and influencing the synthesis of RNA and proteins. At the same time, cytokinins activate the plasmalemma ATPase and the proton pump, thus influencing the intracellular pH and the membrane potential of cells [49, 50].

In plant cells, cytokinins either are synthesized de novo or are produced indirectly from the degradation of tRNA molecules [51, 52]. In the first case, cytokinins are formed by the condensation of dimethylallyl pyro-

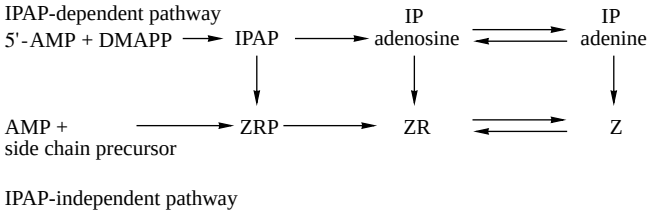

Fig. 1. Two pathways of the de novo biosynthesis of cytokinins (DMAPP, dimethylallyl pyrophosphate; AMP, 5'-adenosine monophosphate; IPAP, isopentenyl adenosine-5'-monophosphate; IP adenosine, isopentenyl adenosine; IP adenine, isopentenyl adenine; Z, zeatin; ZR, zeatin riboside; and ZRP, zeatin riboside 5'-monophosphate).

phosphate (DMAPP) and 5'-adenosine monophosphate (AMP) into isopentenyl adenosine-5'-monophosphate (IPAP), which then transforms into isopentenyl adenosine (IP adenosine), isopentenyl adenine (IP adenine), zeatin riboside (ZR), and zeatin (Z) (Fig. 1). The key enzyme of this pathway, isopentenyl transferase, was isolated and partially purified from plants and some microorganisms. The enzyme has a molecular mass of about 27 kDa, an optimum pH of about 7.0, requires Mg^{2+} ions, and is specific to DMAPP and AMP. Genes responsible for the de novo synthesis of cytokinins were studied only in microorganisms. In *Agrobacterium tumefaciens*, the *ipt* (isopentenyl transferase) and *tzs* (*trans*-zeatin secretion) genes are located in the Ti plasmid. The *ipt* gene, which is under the control of an eukaryotic promoter, is expressed only in host plants, whereas the *tzs* gene is expressed in agrobacterial cells. The *pzs* gene, isolated from the *Pseudomonas savastanoi* plasmid was found to be highly homologous to the *tzs* gene. Astot *et al.* described an alternative pathway of the de novo synthesis of cytokinins (Fig. 1), in which the side chain of cytokinins is synthesized through terpenoids [53].

The indirect production of cytokinins occurs through the isopentenylation of the adenine base located in the anticodon loop of some tRNAs. This reaction, in which mevalonic acid serves as the source of the isopentenyl group, is catalyzed by the tRNA isopentenyl transferase encoded by the *miaA* gene. The cytokinins thus produced are free. The degradation of tRNAs primarily gives rise to inactive *cis*-isomers of zeatin, whereas the de novo synthesis of cytokinins usually gives rise to active *trans*-isomers of zeatin. Chen [51] and Morris [52] also described the formation of *trans*-zeatins during the degradation of tRNA.

In vivo experiments, the effect of PPFM on the germination of seeds and the development of plants was analogous to the effect of cytokinins or the culture liquid of methylobacteria. Using immunoaffinity chromatography, HPLC, and enzyme-linked immunosorbent techniques, Long *et al.* [54, 55] showed that the PPFM isolated from soybean, maize, barley, and *Arabidopsis* plants contain zeatin and zeatin riboside in amounts of 50 to 400 ng/g dry biomass. The polymerase chain reaction (PCR) analysis of the facultative methylotroph *M. extorquens* allowed the isopentenyl transferase gene to be detected.

In our experiments, the bacterium *M. mesophilicum* isolated from the leaf surface of the rye grass *Lolium perenne* L. and the nonpigmented obligate methylobacterium *M. mays* isolated from the leaf surface of the maize *Zea mays* L. were found to be able to synthesize cytokinins. Using a biotest with the *Amarantus caudatus* L. seedlings, we detected cytokinin activity both in the culture liquids of these bacteria and in individual substances isolated from these culture liquids. Chromatographic and enzyme immunoassay analyses confirmed the presence of zeatin and/or zeatin riboside in these culture liquids and the presence of cytokinins in the culture liquids of the two methylobacteria [56] mentioned above and some other pigmented and nonpigmented methylobacteria and methanotrophs.

At present, the nucleotide sequences of genes responsible for the production of cytokinins in various phytopathogenic bacteria are known. We compared the nucleotide sequences of the *tmr* and *tzs* genes of *A. tumefaciens* and the *ptz* gene of *P. syringae* pv. *savastanoi* known from the literature [57, 58] and the predicted amino acid sequences of the encoded proteins and found that the 5'-terminal DNA sequences of these genes and the corresponding *N*-terminal amino acid sequences of their products are highly homologous. The C-terminal amino acid sequences and the corresponding DNA regions were very variable. However, amino acid residues at positions from 25 to 38 and from 92 to 104 were conservative and those at positions 31–38 and 96–104 were identical [57]. To screen the collection of methylotrophic bacteria for the presence of cytokinin genes by the PCR technique, we synthesized two primers coding for the amino acid residues 31–38 and 98–104 of the Tzs protein and found that the genomes of most collection cultures of methylobacteria and methanotrophs belonging to the α, β, and γ subclasses of the Proteobacteria *Methylobacterium*, *Methylovorus*, *Aminobacter*, *Methylopila*, *Methylarcula*, *Xanthobacter*, *Paracoccus*, *Blastobacter*, *Hyphomicrobium*, *Methylophilus*, *Methylobacillus*, *Methylomonas*, *Methylobacter*, *Methylosinus*, and *Methylocystis* contain nucleotide clusters homologous to the genes responsible for the synthesis and secretion of cytokinins [59].

A more detailed analysis of the *M. mesophilicum* and *M. mays* genomes showed that the sizes of the DNA fragments amplified with the aforementioned primers corresponded to the predicted ones. In addition, electrophoretograms contained nucleotide bands whose size was twice as large as predicted. These data suggest that the genes under discussion are duplicated. This suggestion was confirmed by the repeated amplification of the DNA fragments with the same primers. Such gene duplication is fairly common to microorganisms and probably serves to enhance gene functioning. The expression of the genes in the methylobacteria was

studied at the level of mRNA, since the presence of an mRNA is an indication that the corresponding gene is functionally active. The expression of the genes responsible for the synthesis and secretion of cytokinins was established by the reverse transcription technique followed by PCR analysis. The presence of respective mRNAs in the *M. mesophilicum* cells grown either on methanol or succinate confirmed the constitutive nature of the cytokinin genes [56].

The ability of many plant-associated microorganisms, including methylotrophs, to synthesize cytokinins led Holland to an original hypothesis that the cytokinins are synthesized by the microorganisms and not by the plants [60]. This hypothesis is based on the following facts. First, plants do not have genes responsible for the synthesis of cytokinins (at least, this has not yet been unambiguously shown), although all relevant genes were found to be cytokinin receptors. If cytokinins are produced exogenously, the most efficient metabolic regulation by the cytokinins must involve the tissue-specific modification of these signal molecules, as was really shown in studies of the distribution of cytokinin-modifying enzymes. At the same time, the suggestion that cytokinins are synthesized by plants led to a paradoxical situation: these signal molecules are synthesized and inactivated in the same tissues in which they must act. Second, the cytokinins and their genes found in plants may actually belong to plant-associated bacteria, since the axenity of whole plants and their tissues was not controlled in these experiments. Third, gnotobiotic plants are known to require phytohormones for their growth. In accordance with this, methylobacteria stimulated the growth and morphogenesis of inoculated plants most efficiently if the cultivation medium did not contain plant hormones and vitamins. Fourth, the site of cytokinin synthesis in plants remains unknown. Exogenous cytokinins act locally, i.e., they are not mobile substances. The transfer of the *ipt* gene of *A. tumefaciens* to the host plant cells causes their tumorous growth but does not lead to a global action. The immobility of cytokinins disagrees with the hypothesis of their synthesis in plant roots.

Auxins are a group of plant hormones, indole derivatives, which are produced in the apical meristem of plants. One of the most important auxins is indole-3-acetic acid (IAA), which is synthesized from tryptophan. In addition to IAA, plants usually contain other indole compounds, which are either intermediates of the synthesis of IAA or products of its conversion. These are indole-3-pyruvic acid (IPA), indole-3-lactic acid (ILA), indole-3-acetonitrile, indole-3-acetaldehyde, tryptamine, and tryptophol. The auxin activity of indole compounds is due either to their conversion to IAA or to their inherent activity. Like other phytohormones, IAA results in diverse physiological effects in plants: it stimulates the division, extension, and differentiation of plant cells, enhances root formation by promoting the conversion of parenchyma into xylem and phloem, and regulates the leaf fall and fruit ripening [61]. In plant tissue cultures, auxins, together with cytokinins, promote cell differentiation and induce the formation of roots.

Many epiphytic and soil microorganisms are able to synthesize and secrete IAA [62, 63]. Such microorganisms may exert either beneficial effects on plants (these are bacteria of the genera *Azospirillum, Rhizobium*, and *Pseudomonas*) or adverse effects (these are phytopathogenic *Pseudomonas, Agrobacterium*, and *Xanthomonas* bacteria) [62]. Our recent studies showed that many aerobic methylotrophic bacteria are also able to synthesize IAA [19, 64, 65]. In particular, various indole compounds were detected in the culture liquids of 37 methylotrophic bacteria belonging to different taxa [65]. These bacteria secreted from 5 to 120 μg/ml indole compounds into the medium. A more detailed analysis showed that methylobacteria with the serine pathway of C_1 metabolism (*M. mesophilicum* and *Aminobacter aminovorans*), the ribulose monophosphate pathway (*M. mays*), and the ribulose bisphosphate pathway (*Paracoccus kondratievae*) synthesize IAA, ILA, and IPA, respectively. In serine-pathway bacteria, indole-3-acetamide was also detected.

The synthesis of indole compounds in methylobacteria was found to be strongly inhibited by ammonium ions: the substitution of KNO_3 for $(NH_4)_2SO_4$ in the cultivation medium augmented the amount of synthesized indoles by 2 to 15 times, depending on the strain. Earlier, a similar effect of nitrogen sources was shown for bacteria of the genera *Azotobacter* [66] and *Pseudomonas* [67]. The inhibition of indole synthesis by ammonium ions is probably due to their competition with the amino groups of tryptophan eliminated in the process of IAA synthesis. When the initial pH of the cultivation medium was raised to 8.2, the inhibitory action of ammonium ions decreased, presumably because of their conversion into free NH_3.

It is known that tryptophan is the major, if not the sole, precursor of IAA in microorganisms [68]. In our experiments, the addition of tryptophan to the cultivation medium enhanced the synthesis of indole compound by methylobacteria [65]. In *Azospirillum brasilense*, there is a subsidiary, tryptophan-independent, pathway of IAA synthesis. However, in contrast to plants [70], its contribution to the total synthesis of IAA is insignificant [69].

Microorganisms can synthesize IAA through IPA, indole-3-acetamide, tryptamine [71], and indole-3-acetaldehyde. The last pathway involves an oxidase oxidizing the side chain of tryptophan (the so-called tryptophan side-chain oxidase, or TSO) [72]. IAA is synthesized from tryptophan through tryptamine in three steps, of which only the first step (the decarboxylation of tryptophan) is specific to this biosynthetic pathway. It should be noted that tryptophan decarboxylase and TSO have not yet been found in methylobacteria [65].

The two-step synthesis of IAA through indole-3-acetamide, which is catalyzed by tryptophan

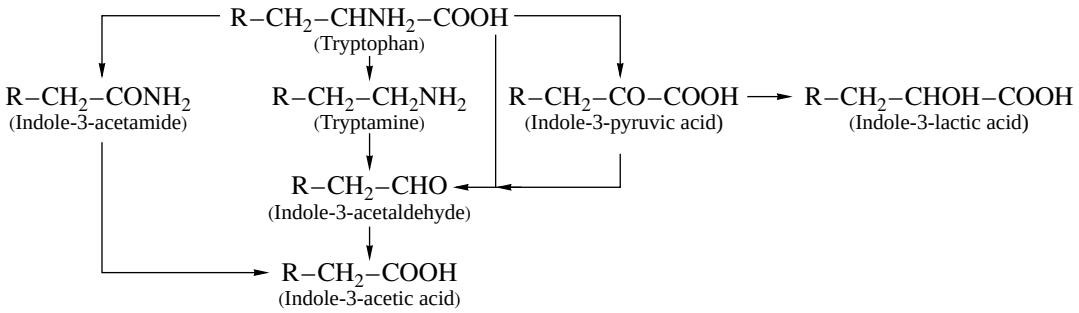

Fig. 2. Biosynthesis of IAA in bacteria.

2-monooxygenase and indole-3-acetamide hydrolase, was first found in the phytopathogenic bacteria *P. syringae* and *A. tumefaciens* and then in *A. brasilense*. Although indole-3-acetamide was detected among the exometabolites of methylobacteria, this fact cannot be considered as convincing evidence that IAA is synthesized just through indole-3-acetamide.

The biosynthesis of IAA through IPA is the major pathway of IAA formation in plants. This pathway involves the transfer of the amino group of tryptophan to IPA (this step is catalyzed by aromatic aminotransferases) and the reaction of decarboxylation with the formation of indole-3-acetamide, which is oxidized to IAA. In methylobacteria, we found several proteins with aminotransferase activity [65]. This finding suggests that these bacteria synthesize IAA through IPA (Fig. 2).

Vitamin B_{12} is a group of complex compounds of a trivalent cobalt, the most important of which is cyanocobalamine with a cyanide group as the cobalt ligand. Other cobalamines may contain other groups as the cobalt ligands; e.g., aquacobalamine and cobalichrome contain -OH and $-NH_4^+$ groups, respectively. Vitamin B_{12} plays an important part in the synthesis of biologically active compounds containing methyl groups, as it serves as the coenzyme in various isomerization and transmethylation reactions. Many plant-associated methylobacteria can accumulate vitamin B_{12}, especially when grown on methanol, in amounts of up to 3 mg/l [73]. Cobamide enzymes, containing the coenzyme form of vitamin B_{12}, were found in the microorganisms that are able to synthesize vitamin B_{12}, as well as in the nitrogen fixing root nodules of legume and alder plants [74]. Many flowering plants, including mono- and dicotyledons, also contain B_{12}-linked enzymes [75, 76], although they cannot synthesize vitamin B_{12} [77] and are dependent on exogenous sources of this vitamin. Exogenous vitamin B_{12} stimulated the growth and development of moss gametophytes, increasing their biomass, amount, length, and the degree of branching [78, 79]. The effect of the exogenous vitamin B_{12} was analogous to that of the PPFM grown in association with the moss *S. nemorosa* under laboratory conditions [9], suggesting that the plant growth–stimulating effect of PPFM is due to their ability to synthesize vitamin B_{12}. These findings explain the aforementioned data suggesting that the stimulating effect of methylobacteria on the growth and morphogenesis of tobacco, potato, and fiber flax plants was especially profound if their cultivation media did not contain vitamins and phytohormones [38].

Exopolysaccharides. Many methylotrophs can synthesize exopolysaccharides (EPSs) [80], which play an important part in the formation of bacterial colonies on the surface of host plants [15]. According to the observations of Oleskin *et al.* [81], a microbial population begins to interact with the host plant when its population density becomes sufficiently high. In colonies, the envelopes of microbial cells (capsules and extracapsular slime) merge to form a biopolymeric matrix composed of acidic polysaccharides and glycosylphosphate-containing biopolymers, such as teichoic acids and glycoproteins. Structurally, the matrix represents a system of microtubules intended for the movement of cells and the transport of various substances. The matrix also performs a protective function, preventing constituent cells and the whole colony from dehydration, extreme temperatures, the action of hydrolytic enzymes, UV radiation, etc. Some polysaccharide and peptide components of the matrix can act as cryo-, thermo-, and xeroprotectants. The matrix conducts microbial exometabolites and the products of cell autolysis, including signal substances, which are never detected in the culture liquid in noticeable amounts, as they can hardly pass through the cell envelope. The biofilm produced by methylotrophs may promote the transfer of their exometabolites to the host plants and protect the latter from adverse environmental influences.

Osmoprotectants are uncharged low-molecular-weight water-soluble substances, which are nontoxic to cells even at high concentrations. These are some alcohols (glycerol, sorbitol, and mannitol), sugars (sucrose and trehalose), and amino acids (glutamate, betaine, proline, 5-oxoproline, and ectoine). In response to an increased osmolarity of the medium, aerobic halophilic

methylobacteria of the genera *Methylophaga* and *Methylarcula* synthesize glutamate and ectoine, respectively [82]. Halophilic and alkaliphilic methanotrophs can synthesize ectoine, 5-oxoproline, and sucrose [83, 84]. The synthesis of osmolytes by methylotrophic bacteria serves to adapt them to increased osmotic pressures. There is evidence that even nonhalophilic methanotrophs contain ectoine genes, indicating that the ability to synthesize osmoprotectants is typical of many bacteria. Various stresses, such as drought, soil salinity, and low temperatures, limit the growth of plants and their productivity because of the impaired intracellular water balance. Many plants have evolved the ability to synthesize and accumulate osmolytes and cryoprotective proteins (such as osmotin and osmoprotectin) in response to the action of abiotic stresses [85, 86]. The transgenic plants that contain bacterial genes responsible for the synthesis of osmoprotectants were found to be more resistant to water stress than the plants that lack such genes [85]. In nature, the osmoprotectants can be synthesized by soil microorganisms and then transferred to the host plants.

CONCLUSION

Plants have long established symbiotic relations with some microorganisms, such as aerobic methylotrophic bacteria, which are ubiquitous and abundant in the plant rhizosphere and phyllosphere. The symbiotic relationship between plants and bacteria is as yet poorly understood; however, it is clear that this relationship is mutually beneficial for both symbionts. The plants serve as physical substrates and provide nutritive substances (e.g., methanol) for the growth of the bacteria. In turn, the symbiotic bacteria utilize methanol, which is harmful to plants, and return the useful products of its transformation, such as ammonia, to the plants. Methylotrophic bacteria produce EPSs, which help them to maintain their population on the leaf surface and to obtain organic substances from the apoplast. At the same time, the methylotrophs beneficially influence the symbiotic plants, contributing to their nitrogen metabolism, providing them with vitamins and growth-promoting hormones (auxins and cytokinins), and enhancing their resistance to various stresses. It should be noted that the proportion between the auxins and cytokinins provided by the methylotrophs to symbiotic plants is presumably optimal for the proper growth of the plant roots and shoots.

Some problems of the physiological, biochemical, and molecular genetic fundamentals of the symbiotic relationship between plants and PPFM have yet to be solved. In particular, little is known about the exact chemical structure and the pathways by which the methylotrophs synthesize auxins and cytokinins. The experimental data presented in this paper suggest that methylobacteria synthesize IAA via IPA. However, further studies are required to elucidate whether or not alternative pathways are involved in the synthesis of these plant hormones. The PCR analysis of some methylotrophic bacteria showed that their genomes contain nucleotide clusters whose sequence is analogous to that of the *tzs* gene responsible for the de novo synthesis of cytokinins. According to preliminary data, free cytokinins in methylotrophs result from the degradation of tRNA. In this case, zeatin and zeatin riboside are produced in the active *trans*-isomeric form and do not require further isomerization. The ability of the methylotrophs to synthesize other growth-promoting factors has not been studied.

The facts that methylotrophic bacteria were detected not only on the plant surface but also in the plant tissues and that some bacterial enzymes were isolated from the plant cells pose a problem of the possible exchange of genetic information between the symbiotic plants and methylotrophs. It is known that many phytosymbionts can protect the host plants from phytopathogens in a way other than through the synthesis of antibiotics and fungicides. The antagonism between aerobic methylotrophic bacteria and phytopathogenic microorganisms has not been studied either.

The beneficial effect of methylotrophic bacteria on plants can be augmented by improving the existing bacterial strains by the methods of genetic engineering and selection. When choosing the dosage and schedule of the application of fertilizers, it should be taken into account that they must be appropriate not only to plants but also to symbiotic microorganisms. It is noteworthy that methylotrophs enhance the survival of plant seeds during storage and promote their subsequent germination.

The ability of methylobacteria to stimulate the growth and morphogenesis of plants in vitro indicates their promise in experimental plant physiology and biotechnology. The colonization of host plants by the methylobacteria in the absence of competition with unwanted microflora may form a basis for the creation of new technologies, such as the clonal reproduction of plants with a low regeneration capacity. Obligate methylobacteria are obviously more promising for the colonization of gnotobiotic plants than facultative methylobacteria, since the obligate methylotrophs can grow only on C_1 substrates and, hence, will not grow on the nutritionally rich media used for the cultivation of these plants. Further studies of the physiological, biochemical, and molecular genetic aspects of the phytosymbiosis of aerobic methylotrophic bacteria may increase the efficiency of the application of these bacteria in modern plant biotechnology.

ACKNOWLEDGMENT

This work was supported by grants nos. 99-04-48251 and 01-04-06413 from the Russian Foundation for Basic Research.

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