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REVIEW

Phytosymbiosis of Aerobic Methylobacteria: New Facts and Views

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Abstract—This review highlightsrecent findings on the phytosymbiosis of aerobic methylobacteria, including their biodiversity, occurrence, and their role in associations with plants, as well as the capacity for biosynthesis of bioactive compounds (auxins, cytokinins, and vitamin B_{12}) and nitrogen fixation. Future research directions in phytosymbiosis of aerobic methylobacteria during the postgenomics era are discussed.

Keywords: aerobic methylotrophic bacteria, phytosymbiosis, auxins, cytokinins, 1-aminocyclopropane-1-carboxylate deaminase, nitrogen fixation, nodulation, genoproteomics.

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Aerobic methylobacteria utilizing oxidized and substituted methane derivatives (but not CH_4) are widespread in nature, often in association with plants [1, 2]. These associations are permanent and result from the fact that methylobacteria consume methanol released by plants into the environment through leaf stomata [3]. Methanol is formed during demethylation of cell wall pectin under active growth of plant cells. It is a major volatile organic metabolite of plants, its emission into the atmosphere being up to 100 Tg/yr. Plants are therefore the main source of methanol in the biosphere [4]. The association between plants and methylotrophs is mutually advantageous, because methylobacteria stimulate plant growth and development due to production of bioactive substances: phytohormones (auxins, cytokinins) and vitamins [1, 2].

Genoproteomics data obtained in the last decade resulted in a considerably better understanding of the metabolic aspects of physiology of aerobic methylobacteria, including those under association with plants [5–7]. The goal of this review is to analyze and generalize new information on the interaction between aerobic methylotrophic bacteria and plants.

DIVERSITY AND OCCURRENCE OF METHYLOTROPHIC PHYTOSYMBIONTS

Aerobic methylotrophic bacteria successfully colonize mosses, liverworts, lichens, gymno- and angiosperms, including those with different types of photosynthesis [1, 8–11]. All methanol-emitting land plants are probably inhabited with methylotrophs. Pink-pigmented facultative methylotrophs (PPFMs) of the genus *Methylobacterium*, which are typical inhabitants of the phyllosphere, have been studied most comprehensively [10]. The presence of a carotenoid pigment imparts additional UV resistance to methylobacteria growing in the phyllosphere. The presence of *Methylobacterium* spp. on the surface of and inside plant tissues has been shown in many works, and several species of this genus were originally isolated from plants (Table 1). Quantitative assessment of PPFM abundance by the plating technique showed the presence of 10^4–10^8 CFU per gram of crude plant tissue, i.e., on average, 14% of all bacteria revealed in the phyllosphere by this method [10, 25]. Molecular methods of the study of bacterial populations in the phyllosphere (clone library analysis of the 16S rRNA gene PCR fragments, automated ribosomal intergenic spacer analysis (ARISA), proteogenomic approach, and parallel pyrosequencing) confirm that representatives of the genus *Methylobacterium* are one of the dominant groups of epiphytic and endophytic bacteria [25–27].

Several hundreds of bacterial species are known to colonize plant surfaces [28]. It has been reliably shown that representatives of α- and γ-*Proteobacteria* are predominant, their quantitative ratio varying depending on plant species [25–29]. In general, according to recent estimates, the quantity of clones with the rRNA genes of methylobacteria obtained from macerated plant tissues amounts to 20% of the total number of bacterial clones, which is in good agreement with the data obtained by the plating technique [25, 27].

The abundance and structure of *Methylobacterium* populations inhabiting plant surface along with non-methylotrophic bacteria depend more on the habitat than on the plant species [25]. For instance, *Arabidopsis thaliana*, alfalfa, and dandelion plants collected in the same area were inhabited by methylobacterial populations approximately similar in the species structure. On the contrary, plants of the same species

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Species of aerobic methylobacteria originally isolated from plant tissues/plant surface

Name	Class	Source of isolation	Reference
Hansschlegelia plantiphila	*Alphaproteobacteria*	Lilac buds	[12]
Methylobacterium extorquens	*Alphaproteobacteria*	Soil, common plant symbiont	[13]
Methylobacterium mesophilicum	*Alphaproteobacteria*	*Lolium perenne* leaf surface	[14]
Methylobacterium nodulans	*Alphaproteobacteria*	Root nodules of *Crotalaria* legumes	[15]
Methylobacterium oryzae	*Alphaproteobacteria*	Rice stem tissues	[16]
Methylobacterium phyllosphaerae	*Alphaproteobacteria*	Rice leaf tissues	[17]
Methylobacterium platani	*Alphaproteobacteria*	*Platanus orientalis* leaf	[18]
Methylobacterium populi	*Alphaproteobacteria*	Poplar seedlings	[19]
Methylobacterium radiotolerans	*Alphaproteobacteria*	Rice seeds	[20]
Methylobacillus pratensis	*Betaproteobacteria*	Meadow grass	[21]
Methylophilus flavus	*Betaproteobacteria*	Dog rose phyllosphere	[22]
Methylophilus luteus	*Betaproteobacteria*	Coltsfoot phyllosphere	[22]
Methylophilus rhizosphaerae	*Betaproteobacteria*	Rice rhizosphere	[23]
Methylovorus mays	*Betaproteobacteria*	Maize phyllosphere	[24]

growing in different places were inhabited by methylobacterial populations substantially different in composition. At the same time, the effect of host plant metabolites on the structure of population of concomitant methylobacteria is still possible, since various species growing in the same area nevertheless differed in the species composition of the symbiotrophic microbiota [25, 30]. In spite of the similarity of the species compositions of methylotrophs isolated from different plant species in the same habitats, evidence of species specificity of the symbiosis was obtained. For instance, the structure of methylobacterial populations remained stable on plants of the same species growing in the same place during two years of study [25]. In addition, methylotrophs were found in plant seeds [10] and buds [9] and inside plant tissues [31, 32].

The highest quantities of methylobacteria were found on leaf surfaces, especially on the lower side of the lamina containing most of the stomata, which are the main pathway for methanol emission [32, 33]. Indeed, using the methylotrophic yeast *Pichia pastoris* carrying a methanol-sensitive promoter fused with the *gfp* reporter gene, it was established that stomata are the main source of methanol emission from leaf surface. In addition, it was proved that inoculation of plants with methylobacteria resulted in a considerable decrease in the level of methanol released from plants into the atmosphere [32].

Molecular methods were used to show the presence of methylobacteria inhabit not only on plant surface but also within plant tissues. The FISH technique demonstrated the presence of *Methylobacterium extorquens* inside pine buds (*Pinus sylvestris* L.), inhabiting mainly primordial cells and gum ducts [31]. Moreover, the inoculation of alfalfa (*Medicago truncatula* L.) with *M. extorquens* labeled with the green fluorescent protein (GFP) showed that methylobacteria penetrated into the intercellular spaces of the epidermis and, to a lesser extent, of the leaf mesophyll [34].

In spite of the advances in the study of phyllospheric PPFM, proper attention has not been paid to the rhizospheric and nonpigmented methylotrophs–phytosymbionts exhibiting great taxonomic and physiological diversity. Description of the nodule symbiont *Methylobacterium nodulans* forming nodules in legumes of the genera *Crotalaria* and *Lotononis* was an important event [15]. Moreover, the collection of type strains of methylobacteria originally isolated from plant tissues or the plant surface has considerably grown in the last decade (table). Representatives of the genus *Methylobacterium* are predominant among the new taxa of methylobacterial phytosymbionts. This is not surprising, because it is the most representative genus of methylotrophic bacteria (>30 species). The most interesting event was the finding of novel taxa–phytosymbionts represented by obligate or restricted facultative species of the genera *Hansschlegelia, Methylovorus*, and *Methylophilus*. Obviously, due to the peculiarities of C_1 metabolism, obligate and restricted facultative methylotrophs are more closely associated with plants than the facultative ones [2].

THE ROLE OF METHYLOTROPHY IN PLANT COLONIZATION

C_1 metabolism plays the key role in the ability of methylobacteria to colonize plants, as was established in experiments with mutants [33, 34]. Inoculation of *Crotalaria podocarpa* with the *M. nodulans* mutant with the deleted *mxaF* gene encoding the large subunit of methanol dehydrogenase (MDH), which is unable

to grow on methanol, resulted in a decreased number of nodules on the roots, suppressed nitrogen fixation, and decreased plant weight. Moreover, by the fusion of the *mxaF* gene promoter and the *lacZ* reporter gene followed by the transfer of the resultant plasmid into *M. nodulans* cells, the *mxa* genes were shown to be most actively expressed in the bacteroids located in the apical part of a nodule, where tissue growth and, accordingly, emission of the maximum amount of methanol occurred [34].

Similar mutants of *M. extorquens* AM1 colonized alfalfa plants with an efficiency comparable to that of the wild type strain. However, competitive plant colonization by the mutant and parent strains mixed in different ratios resulted in gradual displacement of the nonmethylotrophic strain by the methylotrophic one, which confirmed the important role of C_1 metabolism in alfalfa colonization [33].

The proteogenomic approach demonstrated that, in contrast to MDH encoded by the *mxa* genes, induction of a homologous protein XoxF showing a 50% sequence identity with the MxaF protein occurred during plant colonization by methylobacteria [26]. This was an unexpected discovery, since it was considered that XoxF did not participate in methanol oxidation by methylobacteria, because the *M. extorquens* mutant with the impaired *xoxF* gene exhibited no changes in the phenotype [35]. In addition, methylobacterial cells grown in liquid culture contained 100 times less XoxF protein than MxaF [36]. The purified recombinant enzyme XoxF from *M. extorquens* AM1 is a monomer in contrast to MDH, which consists of MxaF and MxaI proteins forming a heterotetramer ($\alpha_2\beta_2$) [37].

XoxF proved to be necessary for successful plant colonization. Deletions of the *mxaF* and *xoxF* genes resulted in loss of competitiveness of the mutants during plant colonization in the mixture with the wild type strain [37]. The biochemical properties of XoxF-MDH (its ability to oxidize, apart from methanol, also formaldehyde and ethanol, albeit at low rates), provide an explanation for its high content in bacterial cells during epiphytic growth. The presence of XoxF probably extends the metabolic possibilities of methylobacteria, because both the growth rate in the exponential phase and methanol uptake were lower in the mutant than in the wild type strain. In this connection, it is noteworthy to mention the recent system research into the physiology and biochemistry of transition of *M. extorquens* AM1 from heterotrophic (succinate) to methylotrophic (methanol) growth. In particular, this elegant work revealed an accumulation of formaldehyde and formate in the culture liquid due to adaptive rearrangement of metabolism during the lag phase [7]. Since the quantity of methanol released from plants varies during the day and depends on the state of the stomata, methylobacteria have to continuously adapt their metabolism. XoxF present in the periplasm of bacteria is responsible not only for more rapid oxidation methanol arriving in great amounts but also for decreasing the concentration of formaldehyde (a toxic product of methanol oxidation) [37]. Moreover, further investigation is required on the role of XoxF in methanol and formaldehyde oxidation and the regulation of its expression.

SYNTHESIS OF BIOACTIVE SUBSTANCES BY AEROBIC METHYLOBACTERIA

1. Biosynthesis of Cytokinins

The discovery of the ability of methylotrophic bacteria to synthesize cytokinins (a class of phytohormones, adenine derivatives) contributed to the system study of methylotrophs as potential phytosymbionts [1, 2]. Moreover, isopentenyl transferases (the key enzymes of cytokinin biosynthesis) have not been found in plants before finding cytokinins in methylotrophs, and this fact provoked some authors to postulate the inability of plants to synthesize cytokinins [38]. Now, however, plants are known to possess several isoforms of isopentenyl transferases and, consequently, have functioning pathways of specific cytokinin biosynthesis [39, 40]. More detailed information about cytokinin biosynthesis in plants is presented in reviews [41, 42].

It seems that most of methylobacteria can also produce cytokinins, because the promotion of seed germination and plant development by PPFM in experiments in vivo is analogous to the effect of cytokinin solutions or the culture liquid of methylobacteria on plants [1]. However, in contrast to phytopathogenic bacteria with determined pathways for biosynthesis of cytokinins (acting as a pathogenicity factor), the pathways of formation of these phytohormones in methylotrophs were unknown. The available genomic information indicated that methylobacteria had no genes encoding isopentenyladenine transferases similar to the *ipt* or *tzs* genes of agrobacteria. Therefore, it was supposed [43] that methylobacteria could nonspecifically produce cytokinins by way of tRNA hydrolysis to isopentenylated adenine (zeatin). This hypothesis was supported by obtaining an *M. extorquens* mutant, in which deletion of the *miaA* gene encoding isopentenyl-tRNA synthetase completely eliminated its ability to synthesize cytokinins [43]. Apparently, most of the nonpathogenic bacteria use this pathway to produce cytokinins. It should be noted that experimental investigation the effect of inoculation of soybean seeds with the *miaA*-negative mutant of *M. extorquens* did not show any significant phenotypic differences from the wild type strain [10]. It is quite possible that cytokinins formed by methylotrophs determine the phenotypic manifestations other than seed germination, where auxins may participate as well.

2. Biosynthesis of Auxins

Auxins are a class of plant hormones (indole derivatives) formed in the apical meristem of plants. Indole-3-acetic acid (IAA) is considered to be the main plant auxin. Capacity for IAA biosynthesis is widespread among microorganisms. The effect of bacteria synthesizing and excreting IAA (*Azospirillum, Rhizobium*) on plant growth and development may be positive or negative when IAA acts as a pathogenicity factor. The effects of exogenous and endogenous IAA embrace practically all aspects of plant ontogenesis: cell elongation and differentiation, root system development, tropisms, flower development, vascular system development, and fruit ripening [44].

The capacity for IAA production has been found in all higher plants, many algae, fungi, and bacteria. However, in spite of extensive research, IAA biosynthesis is still unclear in many respects. Auxins are synthesized by at least two pathways de novo, via tryptophan (Trp) and its precursors or, probably, via indole [45]. The pathways of IAA biosynthesis in plants and bacteria are considered in more detail in reviews [46, 47].

Although the ability to synthesize IAA has been found in a great number of methylobacteria and methanotrophs [48, 49], the enzymes of auxin biosynthesis in methylobacteria are still poorly studied. It is known that the key enzymes of IAA biosynthesis are indole-3-pyruvate decarboxylase, tryptophan decarboxylase, tryptophan-2-monooxygenase, and tryptophan side-chain oxidase [1].

The availability of the complete genome sequence of *M. extorquens* AM1 made it possible to search for the genes encoding the key enzymes of IAA biosynthesis in the genome of this model methylotroph [50]. This search revealed an open reading frame (ORF) of the gene encoding thiamine pyrophosphate (TPP)-dependent decarboxylase of α-ketoacids showing similarity to indolepyruvate decarboxylases (IpdC). Cloning of the gene designated as *ipdC*, as well as purification and characterization of the hexahistidine recombinant enzyme, confirmed its ability to decarboxylate benzoyl formate at the highest rate (k_{cat}), although the maximum specificity constant (k_{cat}/K_m) was observed in the reaction with indolepyruvate. Hence, it was possible to classify the enzyme as an indole-3-pyruvate decarboxylase. The *ipdC*-gene knock-out mutant of *M. extorquens* grown in the presence of tryptophan synthesized 54% less IAA than the wild type strain. Complementation of the mutation resulted in enhanced IAA content in the culture liquid compared to the mutant.

These data are inconsistent with the results of experiments with the rhizosphere bacterium *Azospirillum brasilense*, which exhibited a 90% decrease in IAA concentration under inactivation of the *ipdC* gene. Hence, it follows that IpdC is the key—and, probably, the only—enzyme of auxin biosynthesis in *A. brasilense* [51, 52]. On the contrary, mutation analysis of the phytopathogen *Pantoea agglomerans* revealed the presence of both indolepyruvate and tryptamine biosynthetic pathways for auxins [53]. *M. extorquens* AM1 probably also implements several pathways of IAA biosynthesis, but their role still has to be elucidated. The absence of IpdC-coding gene in the genome of *M. nodulans* also indicates great diversity of IAA biosynthetic pathways in bacteria of the genus *Methylobacterium* [50].

It is notable that IAA is presently considered a signal molecule in bacteria, which was verified experimentally [47]. For example, for *Escherichia coli* it was shown that IAA may be a signal molecule coordinating the behavior of bacteria and thereby enhancing their resistance to unfavorable environmental conditions. IAA was found to initiate the expression of the genes associated with survival under stress conditions. Moreover, the genes encoding the enzymes of central metabolism (tricarboxylic acid cycle, glyoxylate bypass) and amino acid biosynthesis were shown to be regulated by IAA positively, while the *adhE* gene was regulated negatively [54, 55]. We have shown that the metabolism of methylobacteria could also be regulated by IAA, since the addition of auxin to the *M. extorquens* AM1 growth medium resulted in enhanced activity of the enzymes of C_1 and central metabolism, especially in the Δ*ipdC* mutant of *M. extorquens* with impaired IAA biosynthesis [56]. In view of the fact that *M. extorquens* can synthesize IAA, this compound may serve as an autoregulator of bacterial metabolism during epiphytic growth.

3. Influence of Methylobacteria on Ethylene Content in Plants

One of the possible mechanisms of bacterial effect on plant growth and development is their ability to decrease the level of ethylene in plants due to the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase [57]. Ethylene plays an important role in root system development, nodulation, plant aging, exfoliation, fruit ripening, and stress signal transduction [58].

In higher plants, ACC synthase hydrolyzes S-adenosylmethionine into ACC and 5'-methylthioadenosine. In the subsequent reaction, ACC oxidase transforms ACC into ethylene, carbon dioxide, and hydrogen cyanide [59]. In turn, bacteria utilize ACC through the action of ACC deaminase catalyzing ACC hydrolysis to α-ketobutyrate and ammonium ions (Fig. 1) [60]. The ACC deaminase (*acdS*) gene and the relevant enzyme activity were found in many plant-associated and taxonomically diverse bacteria [57]. Until recently, it has been considered that ACC deaminases occur only in microorganisms. However, plants were also shown to contain this enzyme [61].

Bacteria with ACC deaminase stimulate plant growth by decreasing the concentration of ethylene.

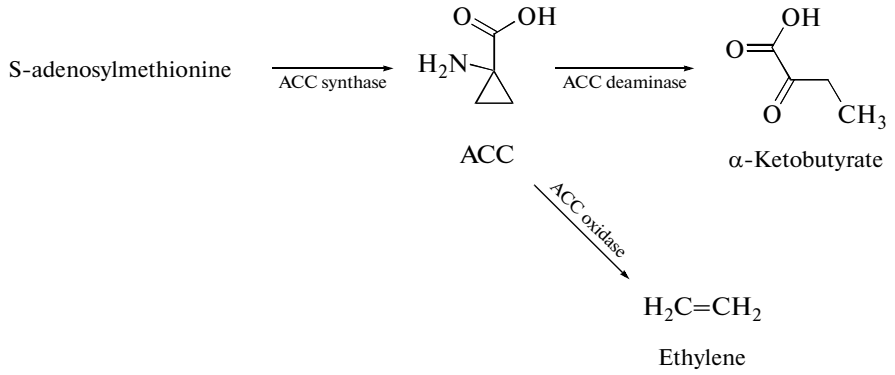

Fig. 1. Metabolic pathways of 1-aminocyclopropane-1-carboxylic acid (ACA).

Ethylene is known to be released by plants under unfavorable conditions (temperature, salt, and acidic and water stresses). Therefore, the inhibition of plant growth is partially or completely relieved after inoculation of plants under stress conditions with ACC deaminase-containing bacteria. Finally, ethylene participates in formation of the so-called induced system resistance of plants to phytopathogens. Although bacteria decrease the level of ethylene in plants, the ability to cause such resistance remains [57].

Inoculation of turnip plants (*Brassica campestris*) with *M. fujisawaense* resulted in effects typical of ACC deaminase-containing bacteria: decrease in the level of ethylene released from plants, promotion of root elongation, and decrease of ACC concentration in the seedlings [62]. Similar experiments were carried out with *M. oryzae* [63]. Moreover, the strains of *Methylobacterium radiotolerans* also possessing ACC deaminase were isolated from the rice phyllosphere. The nucleotide sequence of the structural gene *acdS* encoding ACC deaminase is very similar (98%) to the respective gene of *Rhizobium leguminosarum*. Inoculation of rice and tomato plants with the *M. radiotolerans* strains containing ACC deaminase resulted in the usual effects [64].

The genomic sequences of methylobacteria available from GenBank were used to reveal the genes of ACC deaminases in *M. radiotolerans, M. nodulans* ORS 2060, *Methylobacterium* sp. 4-41, and *Methylibium petroleiphilum* [65]. The *acdS* gene from *M. radiotolerans* JCM2831 was cloned in the expression vector. Heterologous expression in *E. coli* cells followed by metal chelate chromatography was used to obtain an electrophoretically homogenous preparation of the recombinant protein AcdS. The *acdS* gene of *M. radiotolerans* was shown to encode ACC deaminase, which uses ACC as the only substrate. The kinetic characteristics, the optima of activity, and the homotetrameric structure of this enzyme were determined as well. It would be interesting to elucidate the distribution of the *acdS* gene, as well as the properties and expression regulation of AcdS, in different methylotrophs.

4. Fixation of Atmospheric Nitrogen by Methylobacteria

Biological nitrogen fixation is a process performed exclusively by diazotrophic prokaryotes, in which molecular nitrogen is reduced to biologically accessible ammonium [66]. Although the ammonium pool in nature may be also supplemented from abiogenic sources (thunderstorms, volcanoes), the contribution of diazotrophs greatly exceeds the influx of NH_4^+ from other sources.

More than 100 genera of prokaryotes are known to fix atmospheric nitrogen, including members of *Archaea*, all classes of *Proteobacteria*, and many other phyla [67]. Methylobacteria are an extremely heterogeneous group, so it would be logical to assume the occurrence of diazotrophs among them. Up to now, among the validly described genera of methylotrophic bacteria, nitrogen fixation was shown only for representatives of *Xanthobacter* and *Beijerinckia* [68, 69]. Capacity for nitrogen fixation was also revealed in *M. nodulans* and its *nifH* gene sequence was determined [15, 70].

In contrast to methylobacteria, dinitrogen fixation in methanotrophs is known for a long time [71]. For a long time, only certain methanotrophs have been considered nitrogen fixers, because of the failed attempts to determine nitrogenase activity and to reveal the *nif* genes in many methanotrophs. However, recent works have convincingly demonstrated that most of the aerobic methanotrophic bacteria of types I, X, and II are capable of diazotrophy [72–74].

We have analyzed [75] 18 collection strains of taxonomically different methylobacteria belonging to 15 species and implementing different pathways of C_1 assimilation and detected the specific PCR products only in *Beijerinckia* and *Xanthobacter*. Consequently, in contrast to methanotrophs, in methylobacteria the ability to fix nitrogen is much rarer. Genomic analysis

confirmed the presence of the *nifH* gene in the genomes of *Methylobacterium* sp. 4-46 and *M. nodulans* ORS 2060, but not in *M. extorquens, M. populi*, and *M. radiotolerans*. Nitrogenase genes are also absent in the genomes of *Methylovorus* sp. SIP3-4, *Methylotenera mobilis* JLW8, and *Methylobacillus flagellatus* KT.

5. Vitamin B_{12} Biosynthesis in Aerobic Methylotrophs

In addition to phytohormones, many methylobacteria can synthesize other bioactive compounds, particularly vitamins. It is known that PPFM synthesize vitamin B_{12} and accumulate it intracellularly, especially when growing in medium with methanol [1]. We found that aerobic methylobacteria of different taxonomic position synthesize vitamin B_{12} (6–800 ng/l). The maximum content of vitamin B_{12} was revealed in representatives of the genus *Methylobacterium: M. extorquens* G10 and *M. mesophilicum* [76]. The requirement for vitamin B_{12} for plants has not yet been proved; however, it is obvious that methylobacteria synthesizing this vitamin can indirectly stimulate plant growth due to the influence of the B_{12}-dependent bacterial community.

AEROBIC METHYLOBACTERIA AS NODULATING SYMBIONTS

The ability of aerobic methylobacteria to form root nodules has not yet been sufficiently studied, although isolation of a novel species, *Methylobacterium nodulans*, from root nodules of the African legume *Crotalaria podocarpa* was reported ten years ago. Originally, the *nodA* gene of *M. nodulans* was amplified and sequenced as evidence of the nodulation ability [15]. Later another research team isolated one more strain of *M. nodulans*, for which the presence of the *nifH* gene (one of the nitrogenase structural genes) was shown, suggesting the possibility of symbiotic nitrogen fixation by this isolate [70].

In recent years, considerable progress has been made in the study of *Methylobacterium* root nodule symbionts. Analysis of the completed project on sequencing of the genomes of *M. nodulans* and *Methylobacterium* sp. 4-46 leads to the conclusion that these bacteria interact with plants by the "classical" rhizobial type [77]. This mechanism was established through identification of the *nodDABCUIJHQ* gene cluster encoding the enzymes for biosynthesis of the nodulation factors and through determination of the structures of these compounds. In spite of the above, the *nod* genes of *M. nodulans* phylogenetically show greater similarity with the sequences of the respective genes of *Burkholderia tuberum* STM678 than with the rhizobial genes [77].

The strains of *M. nodulans* can fix atmospheric nitrogen, although some representatives of this group (*Methylobacterium* sp. 4-46) do not grow on methanol. This may be due to the fact that, in *M. nodulans*, the MDH-encoding *mxa* genes are localized on the plasmid, which is absent from *Methylobacterium* sp. 4-46 [78].

GENOPROTEOMICS OPENS UP NEW ASPECTS OF PHYTOSYMBIOSIS OF METHYLOBACTERIA

At present, the genomes of the following methylobacteria have been sequenced: *Methylotenera mobilis, Methylibium petroleiphilum, Methylobacillus flagellatus*, *Methylovorus* sp. SIP3-4, and *Xanthobacter autotrophicus* Py2 [79–81]. However, for understanding phytosymbiosis of aerobic methylobacteria, it would be most interesting to sequence eight genomes of representatives of the genus *Methylobacterium*: *M. extorquens* AM1 and PA1, *M. dichloromethanicum* DM4, *M. chloromethanicum* CM4, *M. nodulans, M. populi, M. radiotolerans,* and *Methylobacterium* sp. 4-46 [5].

With the first version of the *M. extorquens* AM1 genome, it was possible to carry out an important experiment for comparison of bacterial proteomes during epiphytic colonization and cultivation in a mineral medium. As a result, it was revealed that, under epiphytic growth of methylobacteria, the content of 45 proteins actually increased compared to the bacteria growing in a synthetic medium. The proteins induced under the epiphytic growth of methylobacteria included methanol oxidation enzymes, stress proteins, and proteins of unknown function [6].

In addition, the regulatory protein PhyR was found, which was similar to the σ-subunits of RNA polymerase. Due to a deletion in the *phyR* gene, the bacteria lost their capacity for efficient plant colonization [6]. Further investigations demonstrated that PhyR, being highly conservative, plays the key role in stress response of α-*Proteobacteria*. This protein was shown to be a positive regulator of 246 target genes, many of them being involved in different stress responses of bacteria: *katE, osmC, htrA, dnaK, gloA, dps*, and *uvrA*. Moreover, the involvement of PhyR in *M. extorquens* responses to drying and heat shock, as well as to oxidative, UV, ethanol, and osmotic stresses, was also confirmed experimentally [82]. A model of gene expression regulation with the involvement of PhyR was proposed. According to this model, in the cells under stress conditions, PhyR is phosphorylated and binds the anti-σ factor NepR, which inhibits the activity of the stress σ factor in the absence of stress. As a result of these events, the cells initiate stress response [83].

A similar approach was used to study microbial communities of soybean (*Glycine max*), clover (*Trifolium repens*), and the wild plant *Arabidopsis thaliana*. Proteogenomic analysis of proteins and DNA from the bacteria washed off the plant surface, followed by

comparison with the metagenomic information, produced similar results. For example, the dominant group of epiphytes were α-*Proteobacteria*, the prevailing representatives of which, in turn, are members of the genera *Methylobacterium* (20.2%) and *Sphingomonas* (20.1%). The proteins of *Methylobacterium* found during epiphytic cell growth were represented mainly by methanol oxidation enzymes, while many proteins of *Sphingomonas* were associated with receiving and transportation of different sugars [26].

The key role in interrelations between symbiotic bacteria and host organisms is often played by the "quorum sensing" mechanism, which consists in formation of low-molecular compounds, *N*-acyl homoserine lactones (AHLs), depending on bacterial population density. After a certain AHL concentration in the environment is achieved, the quorum sensing-dependent genes begin to be expressed in bacteria. The mechanism of this sensing provides pathogenic bacteria with the expression of various genes involved in pathogenesis, while bacterial phytosymbionts acquire properties for plant growth promotion [84]. Several types of AHL were identified in *M. extorquens* AM1. They include the known *N*-hexanoyl homoserine lactone (C6-HL) and *N*-octanoyl homoserine lactone (C8-HL) and, in addition, the new *N*-AHL with two unsaturated bonds (*N*-tetradecenoyl homoserine lactone, C14:2-HL) and with one unsaturated bond (C14:1-HL) [85].

The products of two genes found in the genome of *M. extorquens* AM1 show similarity with *N*-AHL synthetases (LuxI). These genes are designated as *mlaI* and *msaI* and are responsible for the biosynthesis of long-chain (C14:2- and C14:1-) and short-chain (C6- and C8-) *N*-AHL, respectively [85]. It is notable that long-chain *N*-AHL are found in the culture liquid only during the methylotrophic growth of bacteria, while short-chain *N*-AHL are found during the growth on methanol and succinate.

Moreover, since the biosynthesis of C14:2- and C14:1-homoserine lactones decreases by 20% in the Δ*msaI* mutant, the quorum sensing systems in methylobacteria are interrelated [85]. The biosynthesis of short-chain *N*-AHL is regulated, in turn, by the product of the *tslI* gene encoding the truncated *luxI* homolog and localized on the plasmid, because deletion of the *tslI* gene results in the loss of capacity for C6- and C8-HL biosynthesis by *M. extorquens* AM1. In addition, the *tslI* product influences the formation of exopolysaccharides [86]. In spite of the existence of mutants in the genes of AHL biosynthesis, there are no reports on the role of the quorum sensing systems in establishment of "metabolic cross-talk" between methylobacteria and plants. On the other hand, the quorum-dependent genes are of great interest, because the analogous genes of other bacterial phytosymbionts often determine the establishment of partnership with a host plant [84].

CONCLUSIONS

As a whole, the study of phytosymbiosis of aerobic methylotrophic bacteria distinctly exhibits tendencies of modern microbiology: investigations at the molecular level, genome sequencing, and analysis of proteomes. These fine methods of analysis have ensured considerable advance in the interpretation of the pathways for primary oxidation and methanol carbon assimilation. At the same time, various molecular approaches proved to be insufficiently informative in the study of the association between methylotrophs and plants, primarily due to the difficulties in working with inoculated plants. Even such a high-resolution approach to comparison of the proteomes of methylobacteria grown in flasks or washed off the surface of inoculated plants showed no substantial difference in the expression of proteins directly participating in plant growth promotion, such as the enzymes for phytohormone biosynthesis [6].

It is quite probable that the maximal stimulation of plant growth and development by the phyllospheric methylotroph *M. extorquens* occurs under other conditions, such as stress impacts or attacks of phytopathogens. The experiments should, therefore, be performed with other strains of aerobic methylobacteria, especially those isolated from plant rhizosphere, such as *M. nodulans*. Moreover, it is not improbable that methylobacterial proteins involved in promotion of plant growth and development can be expressed constitutively, which makes it difficult to apply the proteomic approach for their investigation. In spite of this fact, considerable progress has been made in the investigation of phytohormone biosynthesis by methylobacteria, the key role of methylotrophy in plant colonization has been ascertained, and the regulator protein PhyR specifically induced during the epiphytic growth of these bacteria has been found.

Owing to successful investigation of the composition of microbial communities in situ, the role of methylotrophs in epiphytic associations becomes clearer. Methylobacteria proved to be predominant on plant surface as one of the most numerous groups of prokaryotes [25]. The solved and unsolved problems of interaction between aerobic methylobacteria and plants are summarized in Fig. 2. Plants supply the microorganisms with a habitat and carbon sources (methanol for methylobacteria, sugars and organic acids for heterotrophs), micro- and macroelements. In their turn, methylobacteria and heterotrophs supply the plants with phytohormones (auxins, cytokinins and gibberellins) and other bioactive substances. In addition, methylobacteria and heterotrophs are able to synthesize vitamins (e.g., vitamin B_{12}, pyrroloquinoline quinone, tetrahydrofolate, and methanopterin derivatives) and excrete them during cell lysis, thereby stimulating plant growth. Finally, inoculation by nonpathogenic bacteria results in appearance of the so-called "induced" plant resistance to pathogens.

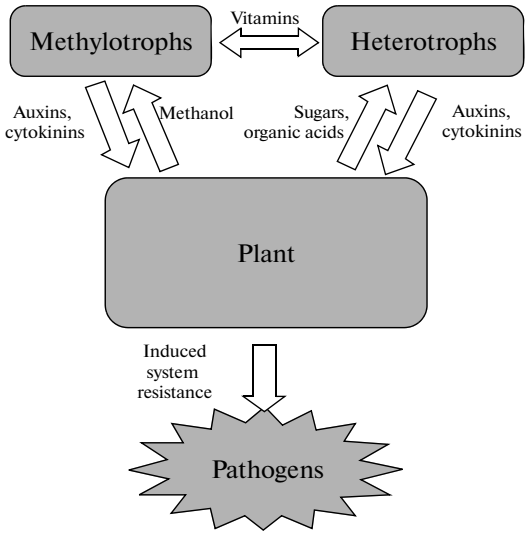

Fig. 2. Achievements and prospects of development of the studies of phytosymbiosis of aerobic methylobacteria.

Based on the experiments on promotion of growth and development of different plants [2, 6, 87] by methylobacteria, particularly of mosses and liverworts [8], the hypothesis of coevolution of methylotrophs and plants was proposed [8]. Prokaryotes supposedly emerged about 3.5 billion years ago, whereas the first eukaryotic cells were a result of ancient symbiosis and were much younger (about 2 billion years). Thus, multicellular organisms such as animals and plants were developing in the environment where bacteria were predominant. When in the early Silurian period (about 400 million years ago), the first mosslike plants began to colonize humid habitats close to rivers and lakes, the bacteria similar to representatives of the genus *Methylobacterium* already existed. Protomethylobacteria associated with ancient mosses probably coevolved together with the host plants. Consequently, this type of interrelation can be considered very ancient, i.e., methylobacteria are the primary phytosymbionts [8].

In conclusion, it should be noted that there are still a number of unsolved questions in the study of phytosymbiosis of aerobic methylobacteria (Fig. 2). For example, it is unclear how closely the methylotrophic and heterotrophic components of the microbiota interact with each other, although positive results are obtained at increased quantity of methylobacteria on plants. In addition, the metabolic and genetic aspects of the interaction between methylobacteria and plants and the nature of the factors involved in the "metabolic cross-talk" between methylobacteria and plants have not yet been sufficiently studied. Except for methanol and AHL, no signal molecules of plants are known that would be analogous to the nodulation factors and could regulate the metabolism of methylobacteria as it occurs under nodular symbiosis. One of the intriguing events in plant physiology and biochemistry is the emergence of induced system resistance to pathogens.

Thus, the recent findings are a basis for using the strains of aerobic methylobacteria as biopreparations stimulating plant growth and development. Further investigation of the physiological, biochemical, and molecular genetic aspects of phytosymbiosis of aerobic methylobacteria will ensure more efficient application of their metabolic potential in modern agrobiotechnology.

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