Review

Potentials, Utilization, and Bioengineering of Plant Growth-Promoting *Methylobacterium* for Sustainable Agriculture

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Abstract: Plant growth-promoting bacteria (PGPB) have great potential to provide economical and sustainable solutions to current agricultural challenges. The *Methylobacteria* which are frequently present in the phyllosphere can promote plant growth and development. The *Methylobacterium* genus is composed mostly of pink-pigmented facultative methylotrophic bacteria, utilizing organic one-carbon compounds as the sole carbon and energy source for growth. *Methylobacterium* spp. have been isolated from diverse environments, especially from the surface of plants, because they can oxidize and assimilate methanol released by plant leaves as a byproduct of pectin formation during cell wall synthesis. Members of the *Methylobacterium* genus are good candidates as PGPB due to their positive impact on plant health and growth; they provide nutrients to plants, modulate phytohormone levels, and protect plants against pathogens. In this paper, interactions between *Methylobacterium* spp. and plants and how the bacteria promote crop growth is reviewed. Moreover, the following examples of how to engineer microbiomes of plants using plant-growth-promoting *Methylobacterium* are discussed in the present review: introducing external *Methylobacterium* spp. to plants, introducing functional genes or clusters to resident *Methylobacterium* spp. of crops, and enhancing the abilities of *Methylobacterium* spp. to promote plant growth by random mutation, acclimation, and engineering.

Keywords: *Methylobacterium*; biofertilizer; biostimulator; biocontrol; plant growth promoting bacteria; microbiome engineering

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1. Introduction

The population is increasing rapidly on Earth, reaching 7.8 billion and is estimated to exceed 9 billion by 2050 (data according to the United Nations Department of Economic and Social Affairs). How to feed the increased population is a major challenge for agriculture. To solve this problem, global food production needs to be increased by 70% to meet demands by 2050 [1]. Therefore, agriculture must be developed in a more sustainable and affordable way. In recent years, chemical fertilizers and pesticides have been used intensively to increase crop yields [2]. Neither chemical fertilizers nor chemical pesticides have been found to be environmentally friendly or ecologically sustainable. Moreover, the application of chemical fertilizers or pesticides always pollutes the surrounding water and air [3]. Chemical fertilizers and pesticides may destroy the soil nutrient balance, lead to poor soil quality, and give rise to a variety of plant diseases [4,5]. Thus, substitutes for chemical fertilizers and pesticides are needed to increase crop yields in a sustainable way. Microbial agents that promote plant growth and yield have the potential to be utilized in agriculture in the future.

Plant microbiomes, which include microbial communities that live, thrive, and interact with tissues such as leaves, flowers, roots, shoots, and seeds, could improve growth, health, and production of plants and protect plants from potential pathogens [6]. Plant microbiomes are composed of bacteria, fungi, and archaea [7,8], among which bacterial microbiomes are regarded to play important roles in promoting plant growth, and could be used in agriculture as microbial agents (<https://www.newleafsym.com/> (accessed on 26 March 2021)). The *Methylobacterium* genus is one of the major components of plant microbiomes. The diversity of the *Methylobacterium* community in soil is influenced by plants growth [9]. The *Methylobacterium* genus was proposed in 1976 and is composed of Gram-negative bacteria belonging to the α -proteobacteria family. Bacteria of the *Methylobacterium* genus generally have pink pigmentation due to carotenoid synthesis. They are able to utilize organic one-carbon compounds (Figure 1), such as formate, formaldehyde, methanol, and methylamine, as the sole carbon and energy source for growing. Most members of the *Methylobacterium* genus are denoted as “pink-pigmented facultative methylotrophs” (PPFM).

Figure 1. Metabolic modules of α -proteobacteria (A) and γ -proteobacteria (B) methylotrophs. For alpha-proteobacteria methylotrophs, it has three interlocked metabolic cycles, that is, the serine cycle, the ethylmalonyl-CoA (EMC) pathway, and the poly-3-hydroxybutyrate (PHB) cycle for C1 assimilation. The overlapped intermediates have been shown. For gamma-proteobacteria methylotrophs, the intermediate pyruvate generated from ribulose monophosphate (RuMP) pathway is decarboxylized into acetyl-CoA, which then enters the tricarboxylic acid (TCA) cycle.

To date, 60 species have been classified in the *Methylobacterium* genus (<https://www.bacterio.net/genus/methylobacterium> (accessed on 26 March 2021)), and 11 species have been classified into a new genus, *Methylorubrum*. As all members of the *Methylorubrum* genus belonged to the *Methylobacterium* genus before 2018, we discuss them together as *Methylobacterium* spp. Some members of the *Methylobacterium* genus dominate the phyllosphere for some plants, especially crops such as rice, clover, and soybean [8]. For example, the strains of *M. extorquens* PA1 and *M. oryzae* were isolated from the phyllosphere of *Arabidopsis thaliana* and rice [10,11]. Other members such as *M. nodulans* and *M. mesophilicum* can inhabit roots, shoots, seeds, and seedlings [12]. Many members including *M. oryzae*, *M. nodulans*, *M. radiotolerans*, and some unclassified species of the *Methylobacterium* genus have been reported to significantly promote plant growth [13–15] (Table 1). Accordingly, *Methylobacterium* spp. are applied, though not widely, in agriculture to increase crop yields in food crops such as wheat, corn, peanut, and tomato (<https://www.newleafsym.com/> (accessed on 26 March 2021)). Here, we mainly review how *Methylobacterium* spp. inhabit plants and promote plant growth, as well as the utilization and perspectives of *Methylobacterium* spp. as microbial agents.

Table 1. Effects of members from the *Methylobacterium* genus on host plants.

Species Name	Host Plants	Effects on Host Plants
<i>M. nodulans</i>	Crotalaria	providing nitrogen; decreasing levels of ethylene [12,16,17]
<i>M. oryzae</i>	Rice	increasing plant cytokinin levels; solubilizing phosphate [13,18–20]
<i>M. extorquens</i>	Pine; Arabidopsis; Brassia	inducing the expression of host defense genes [21,22]
<i>M. mesophilicum</i>	Sugarcane	increasing plant cytokinin levels [23]
<i>M. radiotolerans</i>	Brassia; Combretaceae	providing nitrogen; exhibited high antibacterial and antifungal activity; decreasing levels of ethylene [24,25]
<i>M. populi</i>	Brassia	providing nitrogen [26]
<i>M. komagatae</i>	Brassia	providing nitrogen [17]
<i>M. aquaticum</i>	Brassia	providing nitrogen [17]
<i>M. funariae</i>	Moss	increasing plant cytokinin levels [27]
<i>M. phyllosphaerae</i>	Rice	decreasing levels of ethylene [28]

2. Interactions between *Methylobacterium* and Plants

Generally, bacteria interacting with plants act as endophytes or epiphytes. Endophytic microorganisms inhabit the plant tissue without causing damage or forming any visible external structures to the host [29]. Epiphytes, on the contrary, are those bacteria that live on the surface of plants. *Methylobacterium* spp. interact with plants, usually as both endophytes and epiphytes. *Methylobacterium* spp. acts as an important endophyte in sugarcane and *Jatropha curcas* and play an important role in the growth and yield of these crops [23,26]. Other members of the genus such as *M. extorquens* PA1, *M. oryzae*, and *M. nodulans* were reported to live on the surface of plants in both the phyllosphere and rhizosphere regions [10,20,30]. The rhizosphere, because it comprises the area in the soil around plant roots, is friendlier than the phyllosphere for microorganisms to inhabit. Some species, such as *M. nodulans*, inhabiting the rhizosphere could fix nitrogen and promote plant growth [17]. Microorganisms inhabiting the phyllosphere face harsh environments to survive, including a lack of carbon or energy sources, direct exposure to the rays of the sun, and drought stress.

Although microorganisms in the phyllosphere are less frequently studied than those inhabiting the rhizosphere, in recent decades, as “OMICS” technologies have developed, microorganisms in the phyllosphere have gradually become known. PCR amplification of 16S rRNA genes followed by sequencing of clone libraries and amplicon pyrosequencing have been used to identify bacterial communities in the phyllosphere of *Quercus macrocarpa*, soybean, *Tamarix*, and some tropical rainforest trees and crops as well [31,32]. *Methylobacterium* are detected in the phyllosphere of most plants and are found to dominate the phyllosphere of plants such as rice, clover, soybean, and *Arabidopsis thaliana*, while other α -proteobacteria including *Sphingomonas*, *Pantoea*, *Kocuria*, *Microbacterium*, and *Curtobacterium* genera are also frequently detected [8]. Proteomic and transcriptomic approaches have been used to study adaptation to the phyllosphere of *M. extorquens* [21]. Previous reports showed that methanol released from stomata through cell expansion attracted *Methylobacterium*, which used methanol as the sole carbon source [33]. Compared with the rhizosphere, *Methylobacterium* spp. are found to prefer inhabiting the phyllosphere [34]. *Methylobacterium* spp. produce UV-absorbing compounds that might help the bacteria and plants survive in a phyllosphere exposed to direct sunlight [35]. Members of the *Methylobacterium* genus including *M. extorquens* and *M. mesophilicum*, form biofilms on the plant surface and in tissues, which might help the bacteria survive in plants [36]. When inhabiting plants in the phyllosphere, some strains upregulate the transcription of genes related to methanol utilization to survive in nutrient-limited environments [37,38]. During plant colonization, a methanol dehydrogenase-like protein XoxF was upregulated significantly; proteins involved in formate oxidation, such as Fdh1 and Fdh3, were also

produced more extensively [39]. Strains isolated from plants also show features that help them adapt to the host environment. For example, the *M. extorquens* strain PA1 isolated from *A. thaliana* shows similar genome information to that of the well characterized strain AM1, including genome size, GC contents, and genes involved in methanol utilization. However, these strains showed different growth rates when using different substrates, which might reflect adaptation to the niche from which they were isolated [39]. Moreover, proteomic and transcriptomic studies indicate that, compared with *M. extorquens* strains cultured in the laboratory, those inhabiting the phyllosphere showed obvious adaptation [39]. Some proteins are upregulated in the phyllosphere, especially PhyR, a phyllosphere regulator responsible for general stress [21]. The expression of genes related to anoxygenic photosynthesis was also upregulated, indicating that *M. extorquens* might utilize solar energy to compensate for ATP consumption during growth in the poor carbon source of the phyllosphere [39].

Methylobacterium also provide a major bacterial endophyte community in some plants such as *Brassica*, sugarcane, and *J. curcas* [26,36,40]. *Methylobacterium* spp. were found to account for 69.1% of endophytic bacterial isolates in the leaves of *J. curcas*. In sugarcane, *Methylobacterium* spp. are important endophytes considered responsible for increased seed germination, leaf area, plant height, and number of internodes [36]. These effects might be due to the synthesis of biologically active metabolites such as cytokinins and auxins by *Methylobacterium* spp. *Methylobacterium* spp. including *M. fujisawaense*, *M. phyllosphaerae*, *M. oryzae*, and *M. extorquens* constituted 56% of the bacteria isolated from several Brassian species. *M. fujisawaense* and *M. phyllosphaerae* increased the oilseed fresh weights by 100% and 27%, respectively [40].

Methylobacterial community composition interacting with plants is influenced by many factors including sites, environments, and plant species. Knief et al. [32] studied the influence of the factor site, host plant species, time, and presence of other phyllosphere bacteria on *Methylobacterial* community composition and population size, finding that nearly half of the variation in the *Methylobacterial* community composition could be explained by site and species, with some members of the communities being host-plant specific [41]. In the phyllosphere of some temperate trees, evolutionary associations between groups of bacteria and host species were significant, and bacterial community structure was determined mainly by host species [42].

3. Insights into the Plant Growth Promoting Mechanisms Employed by *Methylobacteria*

The mechanisms used by *Methylobacterium* spp. to promote plant growth have been studied in recent years (Figure 2). Generally, *Methylobacterium* spp. promote plant growth by the following mechanisms:

3.1. Nutrient Acquisition

Members of the *Methylobacterium* genus provide important nutrients to plants as they fix nitrogen and solubilize phosphorus and iron. *Methylobacteria* possess great potential to be utilized as a biofertilizer in agriculture. Many agricultural soils lack one or more nutrients and are not optimal for crop growth [43]. To obtain higher crop yields, chemical fertilizers including nitrogen, iron, or phosphorus are frequently used. Nearly half of the world's food supply depends on chemical nitrogen fertilizers, which might emit greenhouse gas and pollute water during production of the chemical fertilizers. *M. nodulans* isolated from root nodules of *Crotalaria* fixed nitrogen by increasing the number of nodules on the roots of legume plants and promoted their growth [30]. *Methylobacterium* sp. 4-46 and *Methylobacterium* sp. L2-4, which are closely related to *M. radiotolerans*, have also been reported to be involved in nitrogen fixation [44]. In addition, some *Methylobacterium* spp. inhabiting the phyllosphere of plants including *M. populi*, *M. komagatae*, and *M. aquaticum* have been reported to have acetylene reduction activity indicating that they might possess nitrogenase activity [26].

Figure 2. Mechanisms how *Methylobacterium* spp. promote plants growth.

Available phosphorus in soil is always limited for plant growth. Although concentrations of total phosphorus in soils are high, most inorganic phosphorus is bound to calcium, iron, and aluminum or immobilized in phytate, and cannot be utilized by plants [45,46]. To gain an increase in crop production, the available fixed soil phosphorus and applied phosphates need to be solubilized by phosphate-solubilizing bacteria. *Methylobacterium* spp. have the ability to dissolve inorganic phosphates, which in turn promotes phosphate metabolism in both microorganisms and plants [47]. Iron is important for plant growth, while much of the iron in environments is insoluble and not utilized by plants. Bacteria can produce siderophores with low-molecular-mass compounds and high affinity for iron, and accordingly solubilizing iron makes the iron available for plant uptake and promotes plant growth. Tani et al. [39] found that nearly 20% of the strains among 190 strains of the *Methylobacterium* genus isolated from leaf samples of plants produced siderophores [48].

3.2. Modulating Phytohormone Levels

Plant hormones play important roles in plant growth and development. *Methylobacterium* spp. promote plant growth as biostimulators by producing phytohormones such as cytokinins and auxins and by regulating the concentration of ethylene [49]. Ethylene, an important signaling molecule in plants, can regulate cell growth and aging as well as affect the defense system. For example, *M. oryzae* produces 1-aminocyclopropane-1-carboxylate (ACC) deaminase to decrease the accumulation of ethylene by cleaving ACC, the immediate precursor of ethylene [50]. Moreover, *M. oryzae* improves photosynthesis of rice plants in salt [19]. *Methylobacterium* sp. CBMB12, CBMB27, CBMB15, and CBMB20 reduce ACC accumulation in tomatoes by ~50%, and promote tomato growth by 50% to 80%. The reduced level of ethylene also induced pathogenesis-related proteins and defense enzymes, decreasing the disease symptoms of tomatoes treated with pathogens such as *Agrobacterium tumefaciens* and *Ralstonia solanacearum* [51]. Cytokinins are important growth regulators that are responsible for cell division, nutrient allocation, and photosynthetic performance. Members of the *Methylobacterium* genus contain *miaA* genes which are critical to the production of bacterial zeatin, a major type of cytokinins [13]. These *Methylobacterium* spp. increased the cytokinin levels of plants, improved photosynthetic capacity, and ben-

edited the growth of plants [13]. Indole-3-acetic acid (IAA) is the main auxin in plants, playing important roles in root development [52]. *Methylobacterium* can produce IAA, which in turn increases plants IAA concentrations and promotes plant growth [53,54].

3.3. Providing Protection against Plant Pathogens

Studies showed that *Methylobacterium* spp. always protect plants against pathogens by influencing the microorganism community structure or defense system of the plant host [55]. Generally, bacteria induce plant systemic resistance to pathogens by releasing organic compounds or by synthesizing plant cell wall degradation enzymes [56,57]. *Methylobacterium* sp. IMBG290 can induce resistance in potatoes towards *Pectobacterium atrosepticum* by activating the antioxidant system in an inoculum density-dependent manner and by changing the endophyte community [58]. *Methylobacterium extorquens* DSM13060 has also been found to induce the expression of plant defense genes in pine [22]. Recently, *Methylobacterium* spp. was found to be able to protect plants against pathogens directly. *Methylobacterium radiotolerans* MAMP 4754 isolated from seeds of *Combretum erythrophyllum* exhibited high antibacterial and antifungal activity [24]. Vadivukkarasi et al. [59] found that 24 strains of the *Methylobacterium* genus isolated from the phyllosphere of gingers showed antagonistic activity against ginger pathogens including *Pythium myriotylum*, *Macrophomina phaseolina*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, and *Sclerotium rolfsii*. In the future, plant pathogens might be controlled by bioways using *Methylobacteria*.

4. Utilization of Plants Growth-Promoting *Methylobacterium* in Agriculture

Methylobacterium showed great potential as a microbial agent in agriculture. *Methylobacterium* sp. 2A possessed great potential to improve potato crop yield [60]. Tomato and lettuce treated with *Methylobacterium* both showed increases in leaf growth rate, root growth rate, total biomass production, and seed yield and a decrease in cycle time. Agricultural companies have been working on the development of microbial agents that are sustainable and friendly to the world. For example, Indigo Agriculture has developed commercial products composed of microorganisms to improve crop yield. Indigo Wheat improves the yield of wheat by nearly 20%. Indigo Cotton improves cotton yield by 10%, and the increase even reaches 19% under drought conditions. These products show great potential for wide use. Commercial microbial agents composed of only *Methylobacterium* to promote the growth of tomato, cotton, rice, soybean, peanut, wheat, and corn have also been developed by Newleaf Symbiotics, a company developing bioways to improve crop health and yield [61].

5. Engineering of Crop Microbiomes

Plant microbiomes can alter plant physiology and induce resistant systems against pathogens and tolerance mechanisms to stresses such as drought, salinity, and limited nutrients. These functions are carried by one or several microorganisms that form the “core microbiome” [62]. In plants, especially crops such as rice, corn, bean, tomato, and potato, *Methylobacterium* spp. carry these functions and play an important role in microbiomes [15,51,55,63]. Different plants grow in different environments and possess tolerance to different stresses which might depend on microbiomes to some degree [64]. Then, the transfer of these capacities to plants that lack them is important. Several ways have been developed to modify plant microbiomes by introducing microorganisms or microorganism communities including incubating microorganisms with soil, rhizosphere, seeds, or spraying microorganisms on the surface of plants [65,66]. The strategies conducted in recent years are reviewed below, and these strategies might help us identify novel plant growth-promoting *Methylobacteria*, or transfer *Methylobacterium* spp. or their community to plants lacking them (Figure 3).

Figure 3. Strategies for bioengineering of the *Methylobacterium* community to promote crops growth.

5.1. Identification of Novel Plant Growth-Promoting *Methylobacterium*

Although several plant growth-promoting *Methylobacterium* are known to us [11,49,51,54], there might be many more *Methylobacterium* that are not identified but dominate the phyllosphere in many plants. It is urgent to identify more plant growth-promoting *Methylobacterium* due to their potential in agriculture as microbial agents. Host-mediated microbiome selection provides a good way to identify PGPB [67]. Host-mediated microbiome selection is a method that selects microbial communities indirectly through the host or features of the host that influence microbiomes [68,69]. The method has been used frequently to engineer plant microbiomes in the past. Panke Buisse et al. [70] used this method to select on early or late flowering of *A. thaliana*. Plants were inoculated with a starter soil community and grown in sterilized soil. At the end of each growth cycle, flowering time was measured for each replicate; then, soils of the best-performing replicates were chosen to inoculate the next generation of sterilized soils. After ten generations, microbial communities that positively influenced the host trait were selected. Previously wheat rhizosphere microbiomes were engineered in this way and improved the drought tolerance of wheat [69]. Moreover, these novel plant growth-promoting *Methylobacterium* could be identified and classified by combining the approach with “OMICS” technologies.

5.2. Introduce Synthetic Microbiomes to Crops

Methylobacterium spp. with known functions such as plant growth promotion in dry, salty, and nutrient-limited environments could be combined into simple synthetic microbiomes. Introducing the functional *Methylobacterium* community or species to plants lacking them forms synthetic microbiomes for plants and thus promotes plant growth, increases crop yields, and helps plants adapt to different environments. Synthetic microbiomes are usually introduced to plants in two ways: incubation with soils and incubation with seeds or seedlings.

Inoculation of external bacterial strains with rhizospheric soil can change the composition and structure of microbial communities. This method has been used to introduce species including *Methylobacterium* and others. When growing on soil incubated with *Agrobacterium* sp. 10C2, *Phaseolus vulgaris* was reported to show an increase in nodule number and plant biomass. The phosphorus, polyphenols, flavonoids, and total antioxidant capacity of grains also increased significantly. *Agrobacterium* sp. 10C2 promotes *Phaseolus vulgaris* growth by increasing the diversity and changing structure of microbial

communities in the rhizosphere [65]. *Methylobacterium* spp. are also introduced in this way to plants, such as Terrasym 408 (developed by Newleaf Symbiotic) which enhances root development and nutrient uptake of crops.

Incubation with seeds is a popular way to introduce external functional bacteria to plants, either *Methylobacteria* or other bacteria. Commercial products such as Terrasym 401 for soybeans, Terrasym 402 for peanuts, and Terrasym 450 for corn have developed for seed treatment. Recently, Daniel et al. [71] reported that incubation of bacteria including *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 with tomato seedlings promoted tomato growth significantly, with improvement of root biomass and photosynthetic capacity.

6. Engineering of *Methylobacterium* spp. Traits to Enhance the Promotion of Plant Growth

As the microbiomes of plants are influenced by the host, site, and environment etc., introducing external bacteria to plants might pose challenges including how to colonize new hosts and adapt to the environment. Generally, the abilities of bacteria to promote plant growth, protect plants against pathogens, and survive in drought or salty conditions are due to crucial genes or gene clusters (pathways). Introducing functional genes or pathways to resident bacteria might be better than introducing bacteria to plant lacking them as these external bacteria could not usually colonize new plant hosts or could not adapt well to new environments. As the model organism for studying C1 metabolism, *M. extorquens* has been engineered as a microbial cell factory to produce amino acids, organic acids, and alcohols from methanol by introducing heterologous genes and pathways [72–74]. Versatile broad-host-range vectors and insertional expression vectors have been developed for use in methylotrophs with different strengths of promoters driving gene expression [75–77]. Moreover, crucial genes or gene clusters that help bacteria adapt to the host environment or help promote host growth can be identified by using multiomics approaches and genome-scale functional genomic mining [78–81]. Transposon mutation followed by sequencing (TnSeq) and clustered regularly interspaced short palindromic repeats (CRISPR)-based technologies are prominent strategies to screen genes responsible for special functions. Functional genes have been identified in *M. extorquens* by using TnSeq. Recently our colleagues developed an efficient gene mining method using CRISPR interference (CRISPRi) in *M. extorquens*, providing a new way to identify functional genes more efficiently [82]. Introducing these identified genes or gene clusters into resident *Methylobacteria* of plant host will help the resident microbiome obtain external abilities to promote plant growth.

Another choice to enhance the ability of the microbiome to promote plant growth is to improve the ability of resident *Methylobacteria* through random mutations achieved by treating the bacteria with UV light, chemicals, or atmosphere, and using room temperature plasma (ARTP) combined with adaptive laboratory evolution [83].

7. Conclusions and Perspectives

It is important to develop more microbial agents as the demand for agriculture increases significantly. The *Methylobacterium* genus possesses great potential to be utilized as a microbial agent to promote plant growth in different ways. The detailed mechanisms for *Methylobacterium* spp. to promote plant growth and how the bacteria improve crop production are not absolutely clear; therefore more research is necessary to support plant-associated microbiomes in sustainable agriculture. In the future, multiomics approaches will be applied in *Methylobacterium* spp. to characterize methylotrophs and understand how *Methylobacterium* spp. interact with plant hosts and promote their growth. More intense studies might focus on the identification of new *Methylobacterium* species with plant-growth-promoting abilities. With this knowledge, many *Methylobacterium* spp. can be engineered to be more efficient microbial agents and to be more easily introduced to plants, especially crops lacking them.

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