A Survey of *Methylobacterium* Species and Strains Reveals Widespread Production and Varying Profiles of Cytokinin Phytohormones

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Abstract

Background

Symbiotic *Methylobacterium* strains comprise a significant part of plant microbiomes. Their presence enhances plant productivity and stress resistance, prompting classification of these strains as plant growth-promoting bacteria (PGPB). *Methylobacteria* can synthesize unusually high levels of plant hormones, called cytokinins (CKs), including the most active form, trans-Zeatin (tZ).

Results

This study provides a comprehensive inventory of 46 representatives of *Methylobacterium* genus with respect to phytohormone production *in vitro*, including 16 CK forms, abscisic acid (ABA) and indole-3-acetic acid (IAA). High performance-liquid chromatography - tandem mass spectrometry (HPLC-MS/MS) analyses revealed varying abilities of *Methylobacterium* strains to secrete phytohormones that ranged from 5.09 to 191.47 pmol mL^{-1} for total CKs, and 0.46 to 82.16 pmol mL^{-1} for tZ. Results indicate that reduced methanol availability, the sole carbon source for bacteria in the medium, stimulates CK secretion by *Methylobacterium*. Additionally, select strains were able to transform L-tryptophan into IAA while no ABA production was detected.

Conclusions

To better understand features of CKs in plants, this study uncovers CK profiles of *Methylobacterium* that are instrumental in microbe selection for effective biofertilizer formulations.

Background

Most organisms exist in the presence of multiple microbes to the extent that it is essentially difficult to regard them as singular entities. The microbiome describes any collection of bacteria, viruses, protozoa, and fungi – both beneficial and pathogenic – which colonize a host organism. The unique and functional relationship between a host and the specific members of its microbiome forms discrete ecological units, called holobionts (Vandenkoornhuyse et al. 2015). Bacteria which colonize plants inhabiting the root zone (rhizospheric), leaf surfaces (epiphytic), and living within tissues (endophytic), are numerous and diverse (Kutschera 2007). These plant-associated microbiota often play critical roles in plant health, development, and productivity (Arau et al. 2002; Kuklinsky-Sobral et al. 2004; Hardoim et al. 2008; Ryan et al. 2008; Dourado et al. 2015; White et al. 2019). Conserved and optimized through the evolution, the biochemical bases of the interactions between plants and beneficial microbes may provide keys to further understanding and improving plant health in a sustainable manner.

Although the plant microbiome (phytobiome) can be taxonomically diverse, the *Methylobacterium* genus often comprises a significant part of the bacteria present. Generally, *Methylobacteria* are ubiquitous in nature and non-pathogenic to humans or wildlife. They are rod-shaped, obligately aerobic microbes that

can thrive in a wide range of environments including: soil, air, water, and plants (Omer et al. 2004). Most *Methylobacterium* species stain gram-negative and exhibit polar growth, although some exceptions exist (e.g., *M. jeotgali*) (Green 2006). The distinct pink pigmentation of many strains across the genus indicates the presence of specialized carotenoids which likely confer their tolerance to ultraviolet (UV) radiation (Jacobs et al. 2005; Gourion et al. 2008; Yoshida et al. 2017; Kamo et al. 2018) and provide a basis for further classifying individuals as pink-pigmented facultative methylotrophs (PPFMs) (Corpe and Rheem 1989; Yurimoto et al. 2021). Many PPFMs, including *Methylobacterium* strains, are suspected to participate in the development of pigmentation within their host-plants by modulating flavonoid and carotenoid levels within host tissues (Gholizadeh 2012). As facultative methylotrophs, *Methylobacteria* can either use common carbon sources such as monosaccharides, or oxidize a range of single-carbon compounds including: methanol, methylamine, and formaldehyde (Patt et al. 1976; Sy et al. 2005; Kutschera 2007; Šmejkalová et al. 2010; Yurimoto et al. 2021).

Methylobacterium strains can thrive across a wide range of temperatures, salinity, and pH (Green 2006), with certain individuals exhibiting considerable tolerance to chlorine (Hiraishi et al. 1995) and exceptional resistance to gamma irradiation (Ito and Iizuka 1971). *Methylobacterium* have been studied for their suitability in a wide range of biotechnologies including bioremediation of environmental toxins (Aken et al. 2004; Zhang et al. 2008; Ventorino et al. 2014). Most recently, three novel strains of *Methylobacterium* (IF7SW-B2T, IIF1SW-B5, and IIF4SW-B5) were isolated from the international space station (ISS) during flight experiments conducted between 2015-2016; seeming to have evaded decontamination measures and proving suitability even for an oligotrophic environment in microgravity (Bijlani et al. 2021).

Early studies showed that enrichment of the phytobiome with *Methylobacterium* encouraged plant growth and productivity (Holland 1997a, 1997b), leading to the classification of these strains as plant growth-promoting bacteria (PGPB). These first series of discoveries formed the basis of the hypothesis that *Methylobacterium* may be a major source of plant growth hormones known as the cytokinins (CKs) (Holland 1997a). Cytokinins are N^6 substituted adenine derivatives, such as the zeatins (isoprenoid functionality) and topolins (aromatic functionality). As a group, CKs are involved in a wide array of biological functions in plants including: cell division, cell elongation, shoot growth, nutrient uptake, vascular development, and gametophyte development (Sakakibara 2006; Kieber and Schaller 2018; Streletskii et al. 2019; Gibb et al. 2020).

A unique trait of *Methylobacterium* genus that distinguishes it from other PGPB is their ability to biosynthesize unusually high levels of the most active CK forms which includes, in particular, trans-Zeatin (tZ). By producing bioactive CKs that are identical in chemical structure and bioactivity to those endogenously produced by plants, symbiotic *Methylobacterium* can stimulate plant cell division and increase the release of methanol, a by-product of cell wall construction (Nemecek-Marshall et al. 1995; Fall and Benson 1996; Holland 1997b; Šmejkalová et al. 2010). Thus, while the plant is stimulated to grow, *Methylobacterium*, which are able to utilize single-carbon compounds like methanol, receive a stable carbon source that allows them to proliferate (Abanda-Nkpwatt et al. 2006). In this exchange between host and symbiont, *Methylobacterium* have a clear advantage as many other microorganisms in

the phytobiome have more complex habitat requirements and rely on C_6 nutrient sources that are subject to higher competition (Sy et al. 2005). This attribute in itself may prove especially advantageous for *Methylobacteria* in the case of regions with particularly challenging climate and low soil fertility (Biswas et al. 2019), or environments with limited resource bandwidth (space farming).

The production of growth-promoting phytohormones by *Methylobacterium* has propagated interest in uncovering their role as bioinoculants in sustainable crop production systems (Dourado et al. 2015). Studies have gone beyond just illustrating how enrichment of a plant microbiome with *Methylobacterium* increases growth rate, to also demonstrating an increase in tolerance of the host-plant to high salinity (Lee et al. 2015) and drought stress (Jorge et al. 2019). The use of microbes like *Methylobacterium* to support growth and development of crops would be more technically efficient and cost-effective compared to genetic engineering procedures. Moreover, the microbial delivery of growth-promoting phytohormones would be more widely accepted by public consumers relative the more controversial GMO approaches.

The development of a successful bioinoculant begins with the selection of a suitable microbial agent for the target host-plant. Thus far, however, available studies examining CK biosynthesis in *Methylobacterium* are limited in both the number of strains evaluated and the range of CK forms analyzed. Nevertheless, existing research indicates that understanding the types and quantities of the CK forms produced by *Methylobacterium* can help elucidate how selected strains facilitate a beneficial or supportive effect on the host-plant and provide a foundation for novel crop production technologies. This process includes consideration of nutrient and environmental needs; requires no pathogenicity; and has a limited impact on the microbial community native to the host species (Benizri et al. 2001; Esitken et al. 2010; Maneewan and Khonsarn 2017). Furthermore, knowing that there is a well-established understanding of the positive effects that endogenous CKs have in plant systems, uncovering the CK profiles of *Methylobacterium* symbionts may provide an orthogonal variable in the selection of a bioinoculant active agent.

In this study, we expand the knowledge of PGPB potential by producing a comprehensive inventory of 46 *Methylobacterium* strains with respect to their ability to produce and release phytohormones during *in vitro* culture. The analysed strains were specifically selected to capture a wide range of biometric and phenotypic traits, as well as vast differences in sources of origin. Microbial profiles of CK phytohormones, as well as their precursors and derivatives were analysed using the highly sensitive and accurate method of high-performance liquid chromatography positive electrospray ionization tandem mass spectrometry (HPLC-(+ESI)-MS/MS). Furthermore, the analysis of CK secretion by the plant growth promoting strain, *M. oryzae* LMG23582(T), indicates that the hormone biosynthesis is more intense in the media depleted of methanol. To add further phytohormonal context, the bacterial strains were analyzed regarding production of a common CK antagonist, abscisic acid (ABA) through mass spectrometry, and a frequent complementary compound, indole-3-acetic acid (IAA) through a colorimetric method.

Results

Cytokinin Production by Methylobacterium Strains

The collection of 46 *Methylobacterium* strains analysed in this study represented a wide range of growth habitats. Majority of the strains originated from plant organs and soil while some were derived from water, air and other sources (Tab. 1). All bacterial isolates were cultured in a developed for *Methylobacterium*, minimum medium supplemented with methanol as a sole carbon source (DSMZ-125). Use of a minimum medium was essential to ensuring that CKs detected using HPLC-MS/MS were strictly of bacterial origin and not background signal contribution from the constituents of nutrient-rich growth media (Aoki et al. 2021).

Table 1
Inventory of *Methylobacterium* strains evaluated for phytohormone production.

Species	Strain	Isolation Source	Characteristics/Application (as per depository info)
M. aerolatum	JCM 16406(T)	air	None Specified
M. aminovorans	LMG 21752(T)	soil	degrades tetramethylammonium hydroxide
M. aquaticum	B-59286	Phoenix spacecraft surface	None Specified
	DSM 23931	*Arabidopsis thaliana* - silique surface	None Specified
M. bullatum	LMG 24788(T)	greenhouse, cord moss (*Funaria hygrometrica*) - phyllosphere	None Specified
M. cerastii	DSM 23679(T)	*Cerasium holosteoides* - phyllosphere	None Specified
M. extorquens	B-1048	garden soil enriched with sarcosine	taxonomy, transformation host; utilizes alkylamine, formate, oxalate, methanol, methylamine; facultative methylotroph
	JCM 2805	air	taxonomy, genome sequenced strain; facultative methylotroph; used in studies of C1 metabolism; utilizes methanol, methylamine
	NBRC 15687(T)	soil	sterility assurance (antibiotic resistant); utilizes methanol, methylamine, oxalate; facultative methylotroph
	JCM 2806	garden soil, slough	utilizes methanol (methanol oxidaze), methylamine; facultative methylotroph
	DSM 13060	pine (*Pinus sylvestris*) meristem tissue cultures	None Specified
	DSM 23939	*Arabidopsis thaliana* - phyllosphere	None Specified
	JCM 2803	*Psychotria mucronata* - surface sterilized leaf nodules	None Specified
	JCM 20693	mine water	None Specified

Species	Strain	Isolation Source	Characteristics/Application (as per depository info)
M. extorquens	NBRC 103126	soil-litter close to *Rumex* sp.	Degrades oxalate
	NBRC 103127	soil-litter close to *Arum* sp.	Degrades oxalate
	NBRC 103129	soil-litter close to *Eucalyptus* sp.	Degrades oxalate
M. gnaphalii	NBRC 107716(T)	*Gnaphalium spicatum*	None Specified
M. goesingense	DSM 21331(T)	*Thlapi goesingense* - endosphere of the Ni-hyperaccumulating plant	resistance to Ni, Cd and Zn
M. gossipiicola	B-51692 (T)	cotton (*Gossipium hirsutum*) - phyllosphere	produces auxin and ACC deaminase
M. iners	JCM 16407(T)	air	None Specified
M. jeotgali	LMG-23639(T)	traditional fermented seafood (jeotgal)	None Specified
M. marchantiae	DSM 21328(T)	liverwort (*Marchantia polymorpha*) - phyllosphere (thallus)	None Specified
M. mesophilicum	B-14246 (T)	perennial rye grass (*Lolium perenne*) – healthy, green leaves	utilizes methanol, methylamine; facultative methylotroph; antibiotic resistant
	B-2390	household well water	None Specified
M. nodulans	LMG-21967(T)	*Crotalaria podocarpa* - nodules	induces nitrogen-fixing root nodules on legume *Crotalaria* spp.
M. organophilum	LMG-6083(T)	lake water, lake sediment	genetic transformation; enzyme regulation; facultative methylotroph; utilizes methanol, methylamine, trimethylamine; does not utilize methane; produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate
M. organophilum	NBRC 103120	*Ficus elastica* - petiole maceration	degrades oxalate
	NBRC 103121	*Begonia* sp. - petiole maceration	degrades oxalate

Species	Strain	Isolation Source	Characteristics/Application (as per depository info)
M. oryzae	LMG-23582(T)	*Oryzae sativa* cv Nam-Pyeoung - surface-disinfected stem	possesses ACC deaminase activity; produces cytokinins
M. oxalidis	NBRC 107715(T)	*Oxalis corniculata*	None Specified
M. phyllosphaerae	LMG-24361(T)	*Oryza sativa* cv. Dong-Jin - leaf tissues	possesses ACC deaminase activity
M. platani	JCM 14648(T)	*Platanus orientalis* - leaf	None Specified
M. radiotolerans	LMG-2269(T)	Japanese unpolished (unhulled) old and commercial rice grain	radiation resistant; utilizes methanol, methylamine; facultative methylotroph; produces pigment (alpha-bacterioruberin)
	LMG-6379	forest soil	utilizes methanol, methylamine; facultative methylotroph; antibiotic resistant
M. rhodinum	LMG-2275(T)	Alder (*Alnus*) rhizosphere	utilizes methanol, methylamine; facultative methylotroph
M. thiocyanatum	JCM 10893(T)	*Allium aflatuense* – rhizosphere soil	degrades cyanate and thiocyanate
	NBRC 103122	Bryophyllum sp. - petiole maceration	degrades oxalate
	NBRC 103124	*Mesenbryanthemum* sp. - stem maceration	degrades oxalate
M. thiocyanatum	NBRC 103128	soil-litter close to *Mesenbryanthemum* sp.	degrades oxalate
	NBRC 103130	soil-litter close to *Rumex* sp.	degrades oxalate
M. zatmanii	LMG-6087(T)	fermentor operating with formaldehyde as sole source of carbon	utilizes methanol, methylamine, trimethylamine facultative methylotroph
Methylobacterium spp.	LMG-6378	red discolouration of ginned cotton lint	utilizes methanol, methylamine; facultative methylotroph; antibiotic resistant
	DSM 23935	*Cardamine hirsuta* - phyllosphere	None Specified
	DSM 23936	*Medicago truncatula* - phyllosphere	None Specified

Species	Strain	Isolation Source	Characteristics/Application (as per depository info)
	JCM 14673	*Oryza sativa* SC-41 - stem of cultivated rice	None Specified
	JCM 14674	*Oryza rufipogon* W1964 - stem of wild rice	None Specified

Total CK concentration in the cell-free supernatants of *Methylobacterium* strains ranged from 9.9 (*M. platani* JCM14648) to 191.5 pmol mL^{-1} (*M. oryzae* LMG23582) (Fig. 1). Regarding the total CK levels secreted by bacteria, out of the 5 most productive strains (over 100 pmol mL^{-1} CKs), 3 were isolated from plant organs (*M. oryzae* LMG23582(T), *M. phylosphaerae* LMG24361(T), and *M. oxalidis* NBRC107715(T)), while the remaining two (*M. radiotolerans* LMG6379 and *M. jeotgali* LMG23639(T)), originated from the forest soil and the fermented food, respectively.

Of the 28 endogenous CKs monitored, 16 CKs forms were detected at different concentrations (Table S1). The HPLC-MS/MS analysis of CK levels in bacteria supernatants consistently revealed substantial levels of the most active CK-FB forms (mainly tZ, and cis-Zeatin (cZ) in a lower number of strains), as well as relatively high levels of the 2-Methylthiol zeatin conjugate (2MeSZ). In most bacteria strains analyzed, tZ (0.45 – 82.16 pmol mL^{-1}) and 2MeSZ (4.5 – 54.3 pmol mL^{-1}) were the main detected compounds, representing over 70% of the total detected CK content (Fig. 2).

For most of the analysed strains, the main CK group secreted to bacterial supernatants were the most biologically active free bases (CK-FB), followed by methylthiols (2MeS-CKs), and lower levels of ribosides (CK-RB) and nucleotides (CK-NT) (Fig. 3). No considerable differences were observed between the strains regarding the distribution of two most abundant CK groups; however, whenever the ability to produce CK-FBs was more pronounced among the strains, the total 2MeS-CK concentration markedly decreased. Only in the case of *M. oryzae* strain LMG23582(T), *cZ* was detected in higher quantity compared to *tZ* isomer in the cell-free supernatant (146.6 pmol mL^{-1} and 31.5 pmol mL^{-1}, respectively).

Across the inventory of bacteria strains, CK-RB and CK-NT derivatives of isopentenyladenine (iP) were identified at higher concentrations (0.64–6.26 pmol mL^{-1} for isopentenyladenosine (iPR), and 1.41 – 4.61 pmol mL^{-1} for isopentenyladenine nucleotide (iPNT)), relative to the CK-RB and CK-NT derivatives of any of Dihydrozeatin (DHZ), tZ, or cZ, which were detectable at minute levels in the supernatant of *Methylobacterium* strains. Likewise other forms such as DHZ, 2-Methylthio-Zeatin riboside (2MeSZR), and 2-Methylthio-isopentenyladenosine (2MeSiPR) were all only detected at very low levels. Glucoside derivatives and aromatic CKs were not present in the supernatants of any of the *Methylobacterium* strains analysed.

The analysis of CK levels in the supernatants of the most productive strain, *M. oryzae* LMG23582(T), cultured under different concentrations of CH_3COOH (0.25%, 0.5%, 1% and 2%) revealed clear trend in CK

secretion, where the hormone production was intensified by the decreasing concentration on methanol in the growth media (Fig. 4, Table S2).

No endogenous ABA was detected from any of the analysed strains.

Indole-3-Acetic Acid (IAA)

The colorimetric method used in this study to quantify the levels of bacterial IAA revealed that out of the 46 analysed *Methylobacterium* strains, 29 were able to secrete IAA at varying concentrations (0.11-67.47 μmol mL^{-1}) after incubation with an L-tryptophan precursor. Out of the 12 strains originally isolated from soil or rhizosphere, 8 secreted IAA in the concentration ranging from 3.14 to 67.47 μmol mL^{-1} (Fig. 5). Supernatants of two *M. extorquens* strains (JCM2805 and JCM2806) had the highest IAA levels (over 60 μmol mL^{-1}), which corresponded with one of the lowest total CK contents (below 20 pmol mL^{-1}). By contrast, the most active CK producer, *M. oryzae* (LMG23582(T)) did not secrete detectable IAA levels to the culture supernatant, while another strain characterised by high CK concentration in the culture supernatants, *M. phylosphaerae* (LMG24361(T)) released only minute IAA levels (below 4 μmol mL^{-1}). On the other hand, however, *M. thiyocyanatum* (NBRC103122) and *M. radiotolerans* (LMG6379) were associated with relatively high levels of both IAA conversion (over 35 μmol mL^{-1}) and total CKs (over 70 pmol mL^{-1}).

Generally, the levels of IAA converted in *Methylobacterium* cultures from L-tryptophan supplement, were significantly higher compared to the secreted CK levels. However, no endogenous IAA was detected in any cultures that were untreated with L-tryptophan, a direct precursor of IAA biosynthesis.

Discussion

Plant-microbe interactions are complex and variable depending on the relationship between different bacterial strains and plant genotypes. Prior to Holland's hypothesis (1997a) that any biosynthesis of plant cytokinins (CKs) occurred exclusively by the microbial plant symbionts, the investigation of phytohormone production by microorganisms was largely focused on elucidating the mechanisms by which phytopathogens potentiate disease development. These original studies eventually led to the important discoveries like IAA production by *Pseudomonas syringae* and its causal role in initiating tumour-like growths called galls (Akiyoshi et al. 1987; Iacobellis et al. 1994). More recently, the landscape surrounding phytohormone involvement in plant pathogenesis has shifted due to emerging evidence indicating their involvement in the intricate defence mechanisms, related to the host-plant responses to bacterial infections (Choi et al. 2010; Großkinsky et al. 2016) fungi infections (Jiang et al. 2013; Morrison et al. 2015), or attacks by insect predators (Dervinis et al. 2010). Interestingly, many taxa of non-pathogenic microorganisms also produce phytohormones, including bacteria (Shi et al. 2009: Kisiala et al. 2013) and fungi (You et al. 2013; Khan et al. 2015; Streletskii et al. 2019; Bean et al. 2021). The bacteria in question comprise many plant-associated strains of *Methylobacterium* (Ivanova et al. 2000, 2001; Koenig et al. 2002; Meena et al. 2012). Our results confirm that all *Methylobacteria* can produce

CKs, albeit at quite varying levels, which surpass *de novo* production of related hormones (e.g., ABA, IAA) even though they are demonstrably capable of making IAA. Additionally, our data indicate that the intensity of CK secretion *by Methylobacterium* depends on the availability of methanol in the environment.

Pink pigmented facultative methylotrophs (PPFMs) belonging to the *Methylobacterium* genus are a unique group of plant-associated microorganisms known to be considerably beneficial to their hosts (Holland 1997b; Abanda-Nkpwatt et al. 2006; Green 2006; Madhaiyan et al. 2006; Esitken et al. 2010; Meena et al. 2012; Jorge et al. 2019). They are ubiquitous in nature and reside in diverse environments such as leaf surfaces, plant tissues, soil, and in the air. Many plant-endosymbiotic microbes from the *Methylobacterium* genus have been identified as important plant growth-promoting bacteria (PGPB) with natural capability of synthesizing high levels of phytohormones including CKs, which are highly beneficial to the host plant (Holland and Polacco 1994; Ivanova et al. 2000; Dourado et al. 2015). Until now, however, no comprehensive inventory of CK production across a wide breadth of *Methylobacterium* species has been conducted which couples a separation methodology as precise as high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS).

It has been proposed that CKs are produced by *Methylobacterium* as downstream products of tRNA degradation (Koenig et al. 2002). Our work has formed a resource for further understanding the diversity and complexity of phytohormone biosynthesis across CK-producing *Methylobacterium* strains which have already been reported to enhance seed germination, seedling growth, and increase systemic resistance in many plant species (Elbeltagy et al. 2000; Omer et al. 2004; Madhaiyan et al. 2005; Abanda-Nkpwatt et al. 2006; Jorge et al. 2019).

The presence of CKs among various strains of *Methylobacterium* have been reported to date; however, these studies could only detect up to 4 CK forms at a time based on the dated hormone extraction techniques and detection limits (Phillips and Torrey 1972; Sturtevant and Taller 1989; Boiero et al. 2007). Previously, a comprehensive hormone extraction protocol enabled the screening of 25 CK compounds from rhizosphere bacteria with successful detection and identification of 11 forms among *Rhizobium* strains (Kisiala et al. 2013). Using a similar approach, in addition to detecting 16 different CK compounds within *Methylobacterium*, our study illustrates significant variation in the inventory of CK production by different *Methylobacterium* strains compared to other plant-associated microbes - specifically, with respect to the diversity of CKs produced and to the secretion of the highly active tZ free base and its derivatives.

Most widely known for its presence in higher plants, tZ, can bind to at least two plant CK receptors with high affinity and it was characterised as the most active CK in several bioassays (Romanov 2009). In plant systems, tZ participates in basic growth and development (Sakakibara 2006; Osugi et al. 2017), regulation of plant immunity (Großkinsky et al. 2013), or the alleviation of photoperiod stress (Frank et al. 2020). Within the microbial community, previous evaluation of the CK profiles in mycelial biomass of medicinal mushrooms revealed tZ as the most abundant CK form in the majority of species (Vedenicheva

et al. 2018); yet, the regulatory functions of tZ in mushroom growth could not be fully elucidated. By contrast, an examination of CK production by temperate forest soil fungi indicated the complete absence of tZ across all 20 strains investigated (Morrison et al. 2017). In another study involving yeasts from different taxonomic groups and habitats, Z-type CKs were detected at high levels; however, the differentiation between discrete Z isomers was not established, nor was a clear conclusion drawn regarding a possible role of Z synthesis in the growth of yeasts (Streletskii et al. 2019).

Studies involving plant-associated bacteria, including rhizobia, have previously documented the production of mainly cZ- and iP-types of CKs, while our investigation shows that *Methylobacteria* stand apart through their secretion of mostly the bioactive tZ. Comparison of phytohormone profiles of the 46 *Methylobacterium* strains revealed remarkable consistencies in the distribution and levels of CK groups and types, indicating there is a characteristic CK pattern among different species of this genus. In general, tZ was detected in higher abundance relative to cZ levels, with CK-FB forms found at higher concentrations than CK-NT and CK-RB forms. Though our work indicated greater presence of the highly active tZ among the representatives of *Methylobacterium* genus, its isomer cZ was also detectable.

Recent research suggests that cZ forms can have a great agricultural potential; cZ, beyond being involved in plant growth regulation, can also play a crucial role in intermediate responses to stress including infection and herbivory, among other environmental interactions (Schäfer et al. 2015) and the two isomer systems together are thought to offer a dual level of response, as either hard-hitting and brief, or with low activity and longer duration (Hluska et al. 2021). trans-Zeatin was found at high levels in some important experimental plants (i.e., Arabidopsis), while in legumes like chickpea and field pea, cZ was identified as the predominant CK form (Emery et al. 1998; Quesnelle and Emery 2007). Diversity in the relative abundance of CK types across different plant species is also an attribute mirrored in the CK profiles of the examined *Methylobacterium*. It can be suggested that selecting a bacterial strain with a CK production profile similar to that of the target plant can work to enhance the effects of beneficial phytohormones in the host through an additive mechanism. Conversely though, selecting a strain that has a CK profile opposite but complementary to the host plant, may in fact work to achieve positive outcomes through supplementation of hormones which have low endogenous expression with longer duration. Such beneficial effects of supplementation may manifest as improvements in response and tolerance to certain biotic and abiotic stresses and may form the basis for the next chapter of research at the biochemical interface between PGPB and their hosts.

Methylthiol-CKs (2MeS-CKs) were the second most abundant fraction in *Methylobacterium* CK profiles. Methylthiol-CKs are the least characterized CK group as the breadth of their physiological effects and participation in signalling cascades have not yet been even partially elucidated (Gibb et al. 2020). This CK group is often suggested to be mainly of microbial origin, and a modification of the tRNA degradation pathway was proposed as a possible route for 2MeS-CK biosynthesis (Morrison et al. 2017; Gibb et al. 2020) including in *Methylobacterium* (Koenig et al. 2002). High levels of 2MeS-CKs were also detected in other studies involving bacterial symbionts such as *Rhizobia* (Phillips and Torrey 1972; Sturtevant and Taller 1989; Kisiala et al. 2013). Consequently, our findings align with earlier work by establishing a

relatively high production rate of 2MeS-CKs among bacteria strains and consistency of the inverse relationship previously observed between 2MeS-CK production and that of more active forms such as tZ (Kisiala et al. 2013; Gibb et al. 2020).

Riboside (CK-RB) and nucleotide (CK-NT) forms are usually considered to be less biologically active, as CK-NTs are the stable precursors to more active CK forms (Sakakibara 2006; Romanov 2011), and CK-RBs are regarded as more suitable for transport within plants (Kudo et al. 2010; Osugi et al. 2017). On the other hand, Nguyen et al. (2021) contest that CK-RBs have roles beyond transport, including potential direct activity (Nguyen et al. 2021). In our study, both the CK-NTs and CK-RBs were less abundant than CK-FBs across the *Methylobacterium* analyzed. As the less bioactive precursors, CK-NTs are more likely to be retained inside the bacterial cells while their secretion to the supernatant is not expected. Overall, only NT and RB derivatives of iP were detected at higher levels relative to other NT and RB forms evaluated. Microbial isopentenyladenine is synthesised via the isopentenyl-dependent mevalonate pathway (MVA; Fébort et al. 2011) and, thus, the presence of iP-type CKs suggests the presence of this pathway in *Methylobacterium*. Yet, the concentrations of iP in *Methylobacterium* supernatants was several times lower than those of tZ, perhaps because iP is less a stable product of the pathway and can frequently be quickly hydroxylated to tZ by cytochrome P_{450} monooxygenases (Sakakibara 2006), or because of the dominant tZ production via tRNA modification (Koenig et al. 2002).

Glucoside-CK conjugates were not detected in any of *Methylobacterium* strains, and this is consistent with reported evolutionary patterns of CKs in prokaryotic organisms compared to higher plants in which glucosylation is exclusively found (Stirk and van Staden 2010; Kisiala et al. 2013).

One of the advantages of *Methylobacteria* over many other plant-associated microbes is their ability to utilize single carbon compounds, such as methanol, and use them as their sole carbon source. This trait is particularly beneficial under common in natural growth environment, limited availability of other energy-rich molecules like soluble carbohydrates. It has been previously hypothesised that *Methylobacterium* strains release CKs to stimulate plant cell division that results in the production of methanol, the by-product of cellulose metabolism (Holland 1997a; 1997b). Our results strongly support this theory, as increasing the concentration of methanol in the medium from 0.25–2% caused decrease in the levels of tZ and 2-MeSZ in the supernatant of *M. oryzae* (LMG23582(T)) from 40.2 and 37.74 pmol mL^{-1} to 9.88 and 9.38 pmol mL^{-1}, respectively.

Indole-3-acetic acid (IAA) production from L-tryptophan by *Methylobacterium* strains was detected through use of the Salkowski reagent and quantified using a common spectrophotometric technique for measuring IAA production that is of microbial origin (Ivanova et al. 2001; Kim et al. 2017; Gang et al. 2019). Without L-tryptophan supplementation no endogenous IAA was detected; however, with L-tryptophan, the total IAA concentration ranged from 0 to 67.47 µmol mL^{-1}. Among a range of biological functions that IAA participates in (response to light, gravity, ingression of pathogens), it can also stimulate root growth (Schaller et al. 2015). Earlier studies with microorganisms showed IAA producers are often gram-negative microbes (Datta and Basu, 2000). In gram-negative rhizobacterial isolates from

Coleus rhizosphere, IAA production was found at concentrations between 1,312 and 1,484 µmol mL^{-1} using spectrophotometric methods including UV-HPLC and thin layer chromatography (Patel and Saraf 2017). Certain obligate and facultative methylotrophic bacteria, in the presence of L-tryptophan, were reported to produce IAA in the range of 28 – 80 µmol mL^{-1} (Ivanova et al. 2001) which is comparable to the findings of this study.

In our investigation, out of 12 *Methylobacterium* strains of soil origin, 8 were found to have measurable levels of IAA when fed with L-tryptophan. While not all 12 soil-borne *Methylobacteria* could produce detectable levels of IAA, the biosynthesis of this compound does appear to be linked to the strains isolated from the phytobiome (leaves, roots) compared to those without a link to a host-plant (air, water, other sources). Furthermore, although the inverse relationship between CK production and IAA conversion rate was not obvious for all the analysed *Methylobacteria*, the strains capable of secreting the most abundant CK levels (i.e., *M. oryzae* (LMG23582(T)) or *M. phylosphaerae* ((LMG24361(T)) were not converting IAA effectively, and high IAA producers were characterised by the low CK concertation in their culture supernatants. This may indicate high specialization and adaptation of *Methylobacterium* strains for their respective ecological niches.

Conclusions

Herein, we provide the most comprehensive to date, survey of CK production by PPFM of the *Methylobacterium* genus. We have established that *Methylobacterium* species are able to secrete a wide range of the most active CK form, tZ, and this occurred at levels higher than any other PGPB, which could indeed benefit plant hosts. Our data indicate that a detailed bacterial CK profile can be used as an attribute orthogonal to the criteria already existing for the selection of bacteria candidates that are highly effective as plant growth promoters. For example, *M. oryzae* (LMG23582(T)) possesses valuable characteristics that contribute to its usefulness as a PGPB (Indiragandhi et al. 2008) and in conferring tolerance to high salinity stress in plants (Lee et al. 2015; Chanratana et al. 2017). Furthermore, another study supports the hypothesis that microbially-derived CKs play an important role in host-plant metabolism, including growth stimulation, enhancement of physiological parameters, and effective nutrient allocation and water management under drought exposure (Jorge et al. 2019). Retrospectively, the findings of these investigations align with the results presented herein where *M. oryzae* (LMG23582(T)) strain is likely mediating marked growth-promoting effects due to its uniquely high CK production. Additionally, testing the effect of growth medium methanol concentrations on CK production, supported the hypothesis that, in plants, *Methylobacteria* secrete CKs to stimulate cell division and release of methanol that microbial symbionts can subsequently utilize as a carbon source. By contrast, other hormones scanned for (ABA, IAA) were not endogenously detected, unless a precursor was fed to the cells (IAA). In the latter case, IAA conversion capacity from L-tryptophan is inversely related to CK levels among many of the analysed strains.

The use of a multifarious selection strategy supported by knowledge of CK production could help reduce research costs associated with laboratory consumables, equipment, and labour associated with large

scale greenhouse or field-trial experiments by streamlining the process of identifying the most potentially effective strains for bioinoculant development. In future, other high tZ-producing strains of *Methylobacterium* genus should be investigated for growth-promotion and stress-alleviating effects in controlled experiments in plant systems to further elucidate their role in crop protection.

Methods

Bacterial Strains and Growth Conditions

Freeze-dried cultures of 46 *Methylobacterium* spp. strains were obtained from five microbe collections: the Agricultural Research Service (ARS) of the Northern Regional Research Laboratory (NRRL), the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) ["German Collection of Microorganisms and Cell Cultures"], the Japan Collection of Microorganisms (JCM), and the National Institute of Technology and Evaluation's (NITE) Biological Resource Center (NBRC). The strain selection was divided as follows; (NRRL) (ARS) – United States of America (*n* = 5), BCCM/LMG - Belgium (*n* =12), DSMZ – Germany (*n* =8), JCM – Japan (*n* =10), and NITE (NBRC) – Japan (*n* =12). The strains were originally isolated from different biological sources including plant organs (phyllosphere, flowers, roots, moss tissue), soil, water, air, and other materials. The detailed information on strain taxonomy, origin, known characteristics and applications is provided in Table 1. The freeze-dried strains were revived in nutrient-rich R2 broth (VWR, Mississauga, Canada) and maintained as 15% (v/v) glycerol stocks at -80^{o}C. The analysed strains varied in their growth characteristics, such as the intensity of pellet pigmentation (white, orange, pale pink to intense pink), growth rate (5mg – 50 mg pellet in 15 ml culture), or ability to aggregation and biofilm formation (Fig. 6).

Screening for Bacterial Phytohormone Production

For phytohormone analyses, *Methylobacterium* stocks were streaked onto agar plates and liquid cultures were started by suspending the bacterial single colonies with a sterile disposable loop in 50 mL Falcon tubes containing 15 mL of selective minimal medium prepared according to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures) index #125, supplemented with 0.5% CH_3COOH (v/v) as a sole carbon source. Aseptic bacteria cultures were incubated in a rotary shaker (28°C/250 rpm). Supernatant samples were harvested on 5-7 day of the culture, when *Methylobacterium* reached the late exponential/early stationary phase (OD_{600}=0.6-1.2, depending on the strain). To determine cell concentration in the liquid culture, bacterial growth was monitored spectrophotometrically by measuring OD_{600} and confirmed by cell count using a standard serial dilution technique.

To find out if the level of methanol in the growth medium affects the rate of CK production by *Methylobacterium*, the independent batch of *M. oryzae* strain LMG23582(T) was cultured in the 125DSM

media supplemented with increasing concentrations of CH_3COOH (0.25%, 0.5%, 1% and 2% v/v). All other experimental conditions remained the same as described above.

Extraction and Purification of Cytokinins (CKs) and Abscisic Acid (ABA)

A modified protocol for liquid culture medium was used for CK and ABA extraction and quantification (Kisiala et al. 2013). The levels of bacterial phytohormones were measured in 12 mL of the cell-free supernatant obtained after centrifugation (Thermo Scientific, Sorvall ST16; 10 min, 4,696 × g) of harvested *in vitro* cultures. Samples of culture medium that were not inoculated with *Methylobacterium* were subjected to analysis as a negative control, to determine that the CKs detected in the bacteria culture supernatant were exclusively of microbial origin.

The profiles of 28 CK metabolites and ABA were analyzed, including: CK free bases (CK-FBs), their riboside (CK-RBs) and CK-nucleotide (CK-NTs) derivatives, CK-glucoside (CK-GLUCs) and methylthiol conjugates (2MeS-CKs), and select aromatic CKs. The cell-free supernatant samples were mixed with 1 ml of cold (–20°C) modified Bieleski #2 extraction solvent (CH_3OH:H_2O:HCO_2H [15:4:1, vol/vol/vol]) using vortex. Internal standards were added to each sample to enable endogenous hormone quantification through the isotope dilution method. Samples were spiked with 144.7 ng of $^2H_4$-ABA (NRC-PBI, Saskatchewan, SK, Canada) and 10 ng of each of the deuterated internal standard CKs (Table 2) obtained from OlChemIm Ltd. (Olomouc, Czech Republic). As deuterated standards of cis-Zeatin-type CKs were not commercially available at the time of this investigation, the levels of cis-compounds were quantified based on the recovery of the deuterated standards of the corresponding trans-compounds.

Table 2
Cytokinins (CKs) scanned for using liquid chromatography-positive electrospray ionization tandem mass spectrometry in *Methylobacterium* supernatants.

Cytokinin (CK)	Labelled CK Standard
Nucleotides (mono- di- and triphosphates; CK-NTs)	
trans-Zeatin nucleotide (tZNT)	$[^2H_3]$DHZRMP
cis-Zeatin nucleotide (cZNT)	
Dihydrozeatin nucleotide (DHZNT)	
Isopentyladenine nucleotide (iPNT)	$[^2H_6]$iPRMP
Ribosides (CK-RBs)	
trans-Zeatin riboside (tZR)	$[^2H_5]$ZR
cis-Zeatin riboside (cZR)	
Dihydrozeatin riboside (DHZR)	$[^2H_3]$DHZR
Isopentyladenosine (iPR)	
Free Bases (CK-FBs)	
trans-Zeatin (tZ)	$[^2H_5]$Z
cis-Zeatin (cZ)	
Dihydrozeatin (DHZ)	$[^2H_3]$DHZ
Isopentyladenine (iP)	$[^2H_6]$iP
Glucosides (CK-GLUCs)	
trans-Zeatin-O-glucoside (tZOG)	$[^2H_5]$ZOG
cis-Zeatin-O-glucoside (cZOG)	
Dihydrozeatin-O-glucoside (DHZOG)	$[^2H_7]$DHZOG
trans-Zeatin-O-glucoside riboside (tZROG)	$[^2H_5]$ZROG
cis-Zeatin-O-glucoside riboside (cZROG)	
Dihydrozeatin-O-glucoside riboside (DHZROG)	$[^2H_7]$DHZROG
trans-Zeatin-9-glucoside (tZ9G)	$[^2H_5]$Z9G
cis-Zeatin-9-glucoside (cZ9G)	

Cytokinin (CK)	Labelled CK Standard
Dihydrozeatin-9-glucoside (DHZ9G)	$[^2H_5]$DHZ9G
Methylthiols (2MeS-CKs)	
2-Methylthio-Zeatin (2MeSZ)	$[^2H_5]$2MeStZ
2-Methylthio-Zeatin riboside (2MeSZR)	$[^2H_5]$2MeStZR
2-Methylthio-N^6-isopentyladenine (2MeSiP)	$[^2H_6]$2MeSiP
2-Methylthio-N^6-isopentyladenosine (2MeSiPR)	$[^2H_6]$2MeSiPR

Samples were extracted overnight at –20°C and evaporated to dryness at 35°C under vacuum (Model SPD111V; Thermo Scientific, Ottawa, Canada). The extraction residues were reconstituted in 200 µl of 1 M HCO_2H (pH 1.4) for purification and concentration of metabolites using automated Gilson SPE 215 Solid Phase Extraction System (Gilson Inc., Middleton, WI, USA). The hormone-containing fraction was purified and concentrated on a mixed mode, reverse-phase, cation-exchange SPE cartridges (Oasis MCX, Waters, Mississauga, Canada) as described by Dobrev and Kaminek (2002), with modifications (Farrow and Emery 2012). Cartridges were activated with high-performance liquid chromatography (HPLC) grade CH_3OH and were equilibrated using 1 M HCO_2H (pH 1.4). After equilibration, each sample was loaded and washed with 1 M HCO_2H (pH 1.4). Hormone groups were eluted based on the charge and hydrophobicity level. Abscisic acid was eluted first using CH_3OH. Cytokinin nucleotides (CK-NTs) were eluted next with 0.35 M NH_4OH. Cytokinin free bases, CK-RBs, 2MeS-CKs, CK-GLUCs and aromatic CKs were retained on the column based on charge and hydrophobic properties and, therefore, were lastly eluted, using 0.35 M NH_4OH in 60% [vol/vol] CH_3OH. Each collected elution was evaporated to dryness at 35°C under vacuum.

Since intact CK-NTs cannot be analyzed directly with the present method, they were dephosphorylated by overnight incubation with 3 units of calf-intestinal alkaline phosphatase (New England Biolabs Ltd., Whitby, ON, Canada) in 1 mL of 0.1 M ethanolamine-HCl (pH 10.4) at 37°C to form CK-RBs. This detection method of CK-NTs potentially reflects pooled contribution of mono, di- or triphosphates in that the isopentenyl or hydroxylated moiety may be transferred to AMP, ADP or ATP (Quesnelle and Emery 2007). The resulting CK-RBs were evaporated to dryness at 35°C under vacuum. The samples were reconstituted in double-distilled water (dd-H_2O) and were further isolated on a reversed-phase C18 solid phase extraction cartridges (C18/14%, Canadian Life Science, Peterborough, ON, Canada), which were first activated with CH_3OH and equilibrated using dd-H_2O. Samples containing CK-RBs were loaded onto the C18 cartridges and were allowed to pass through the column by gravity. The sorbent was washed with dd-H_2O. Resultant CK-RBs were eluted using 100% CH_3OH and eluents were evaporated to dryness at 35°C under vacuum.

Prior to LC-MS/MS analysis, all phytohormone samples were dissolved in starting conditions solvent (CH_3OH:CH_3CO_2H:ddH_2O [5:0.08:94.92, vol/vol/vol] for ABA and CH_3CN:CH_3CO_2H:ddH_2O [5:0.08:94.92, vol/vol/vol] for CKs) and were centrifuged at 12,320 × *g* for 10 min to remove any solid particles (Thermo Scientific). The supernatants were stored at –20°C until further processing.

High Performance Liquid Chromatography - Electrospray Ionization - Tandem Mass Spectrometry (HPLC-(ESI)-MS/MS) Analysis

Levels of plant growth regulators were measured using high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-(ESI)-MS/MS; QTrap5500; ABI Sciex Concord Ontario, Canada, coupled with Agilent 1100 series HPLC; Agilent, Mississauga, ON, Canada). An aliquot (40 µl) was injected on a Luna C18 reversed-phase column (3 µm, 150 × 2.0 mm; Phenomenex, Torrance, Canada). Abscisic acid was eluted using component A: H_2O with 0.08% CH_3CO_2H and component B: CH_3OH, both with 0.08% CH_3CO_2H, at a flow rate of 0.2 mL min^{-1}. Cytokinins were eluted with an increasing gradient of 0.08% CH_3CO_2H in CH_3CN (A) mixed with 0.08% CH_3CO_2H in ddH_2O (B), at a flow rate of 0.2 ml min^{-1}. For the ABA, the initial conditions of 50% B remained constant for 4 min, then changed linearly to 95% B over 6 minutes. This ratio was held constant for 1 minute before immediately returning to starting conditions and re-equilibrating for 20 minutes. The initial conditions for CK groups were 5% A and 95% B, changing linearly in 17 min to 95% A and 5% B. Conditions remained constant for 5 min, and then, immediately returned to initial conditions for 18 min of re-equilibration.

Abscisic acid was analyzed in negative ionization mode and CKs were analyzed in positive ionization mode. Phytohormones were identified based on their analyte specific retention times and multiple reaction monitoring (MRM) channels. All data were analyzed with Analyst 1.6.2 software (AB SCIEX, Concord, ON, Canada). Hormone concentrations were established according to isotope dilution analysis upon direct comparison of the endogenous analyte peak area against the recovered internal standard (Farrow and Emery 2012). Quantification of cZ-type CKs was performed relative to the recovery of labeled tZ-types and retention time of unlabeled cZ standards.

Indole-3-Acetic Acid (IAA)

For the evaluation of the IAA production capacity, 46 strains were grown in the dark in R2 broth supplemented with L-tryptophan (2.5 mM) and in L-tryptophan-free medium. Seven-days-old cultures that were in late exponential phase, were subjected to quantitative screening for IAA production via colorimetric method using Salkowski's reagent (Gordon and Weber, 1951). Obtained by centrifugation (10 min, 4,696 × g), cell-free suspensions of each strain (1 vol) were incubated for 30 min (RT) with Salkowski reagent (1 ml 0.5M $FeCl_3$ in 50 ml of 35% $HClO_4$) (0.5 vol). Indole-3-acetic acid concentration was quantified spectrophotometrically at wavelength 540 nm. The spectrophotometer was calibrated against a blank media with L-tryptophan mixed with the Salkowski reagent. To determine the sensitivity and the

operating range of IAA concentrations in bacterial cultures, a calibration curve was calculated based on dilution series of authentic IAA (0-570 µmol mL^{-1}) (Sigma-Aldrich, Inc., St. Louis, MO, USA).

Statistical Analysis

The experiments evaluating CKs, ABA, and IAA production by *Methylobacterium* were performed in three replicates for each of the tested strains. The levels of bacterial phytohormones were expressed as per pmol or µmol in ml of the culture supernatant (n = 3 ± SE). Blank media samples (control) were processed and analysed using the same methodologies to account for any background levels of phytohormones.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and materials All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests The authors declare that they have no competing interests.

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Authors' contributions AK and RJNE contributed to the study conception and design. Material preparation, data collection and analysis were performed by AK and GLJ. The first draft of the manuscript was written by DP and GLJ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

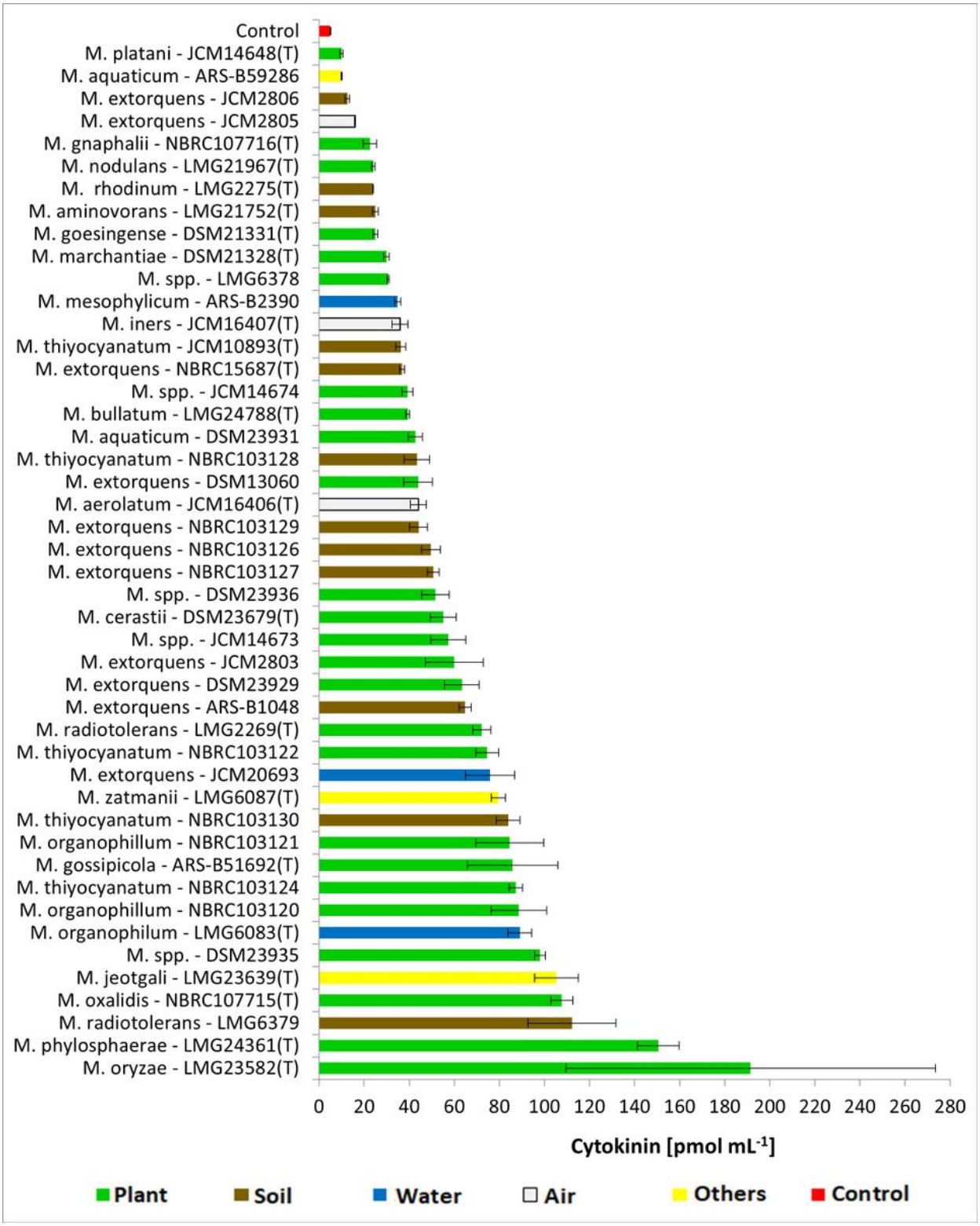

Figure 1

Total cytokinin (CK) concentration (pmol mL-1) detected by HPLC-(ESI+)MS/MS in the cell-free supernatants of 46 Methylobacterium strains cultured in vitro in the DSM125 minimal medium. Cytokinin production was tested in the early stationary phase. Values are means ± SE of 3 replicates.

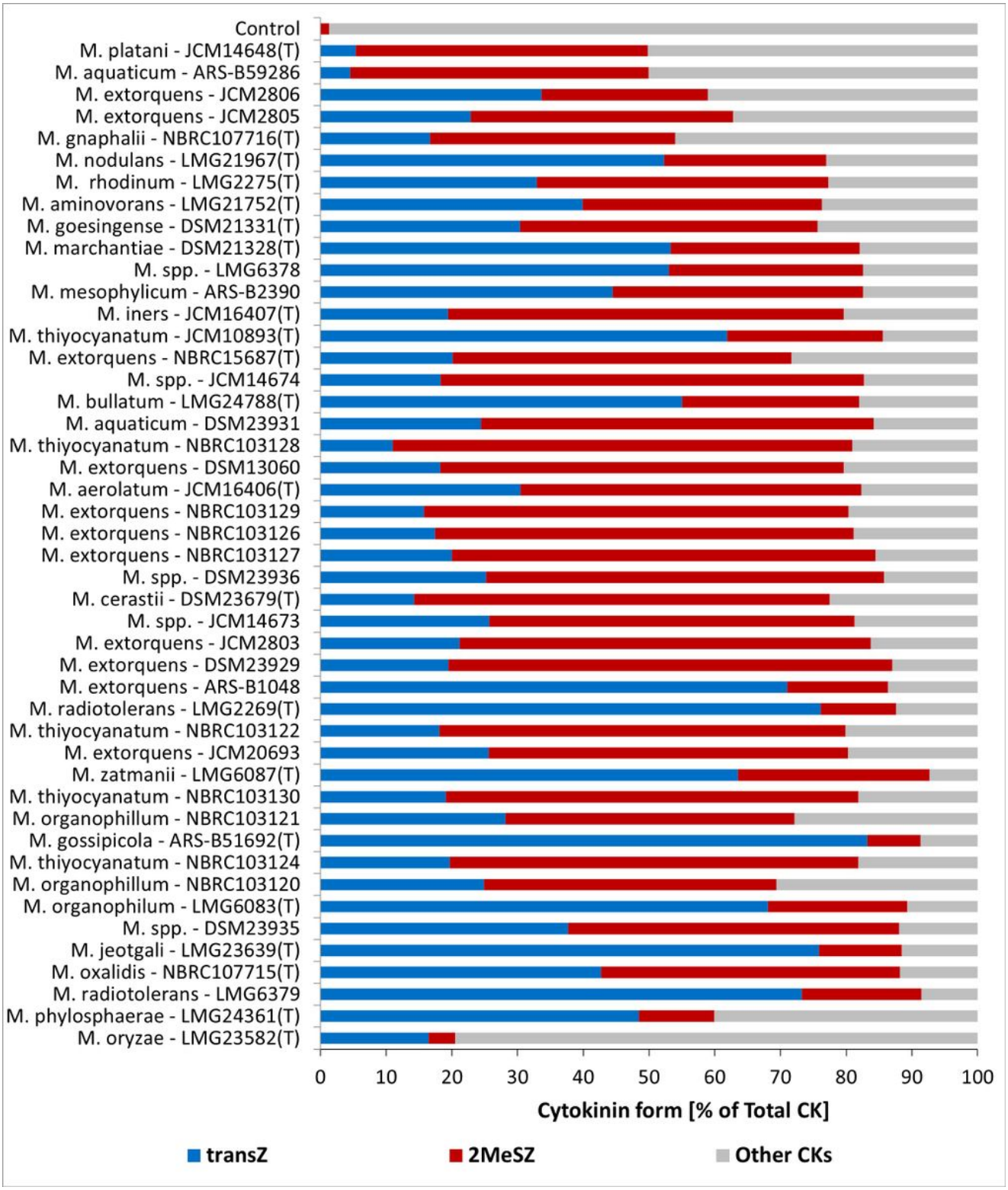

Figure 2

trans-Zeatin (transZ), 2-methylthio-Zeatin (2MeSZ), and other cytokinin (CK) forms reported as percentage of total CK content in the cell-free supernatants of 46 Methylobacterium strains cultured in vitro in the 125DSM minimal medium. Cytokinin production was tested in the early stationary phase. Strains are ordered according to the increasing total CK production (n=3).

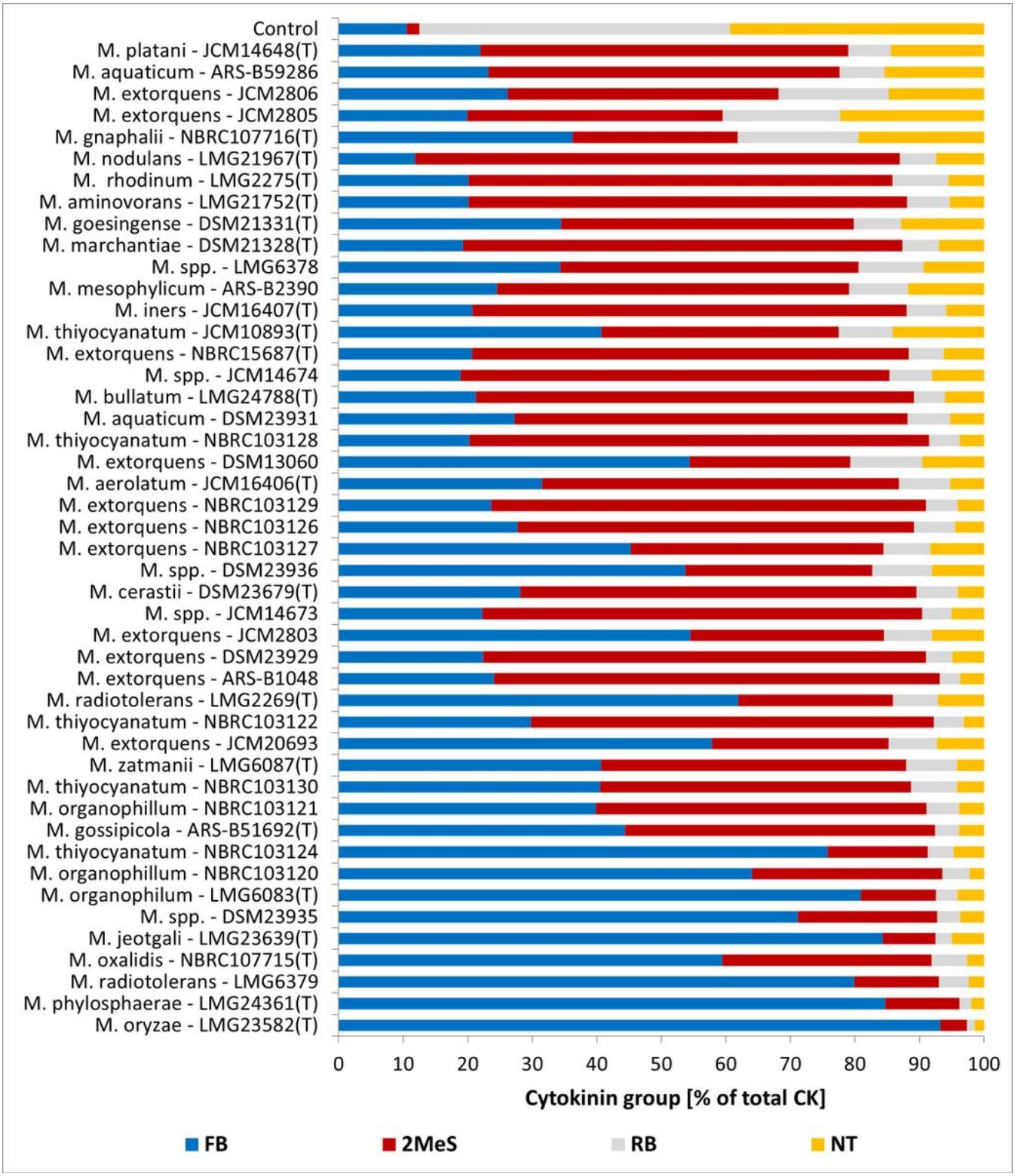

Figure 3

Cytokinin (CK) fractions: free bases (FB), methylthiols (2-MeS), ribosides (RB), and nucleotides (NT) reported as percentage of total CK content in the cell-free supernatants of 46 Methylobacterium strains cultured in vitro in the DSM125 minimal medium. Cytokinin production was tested in the early stationary phase. Strains are ordered according to the increasing total CK production (n=3).

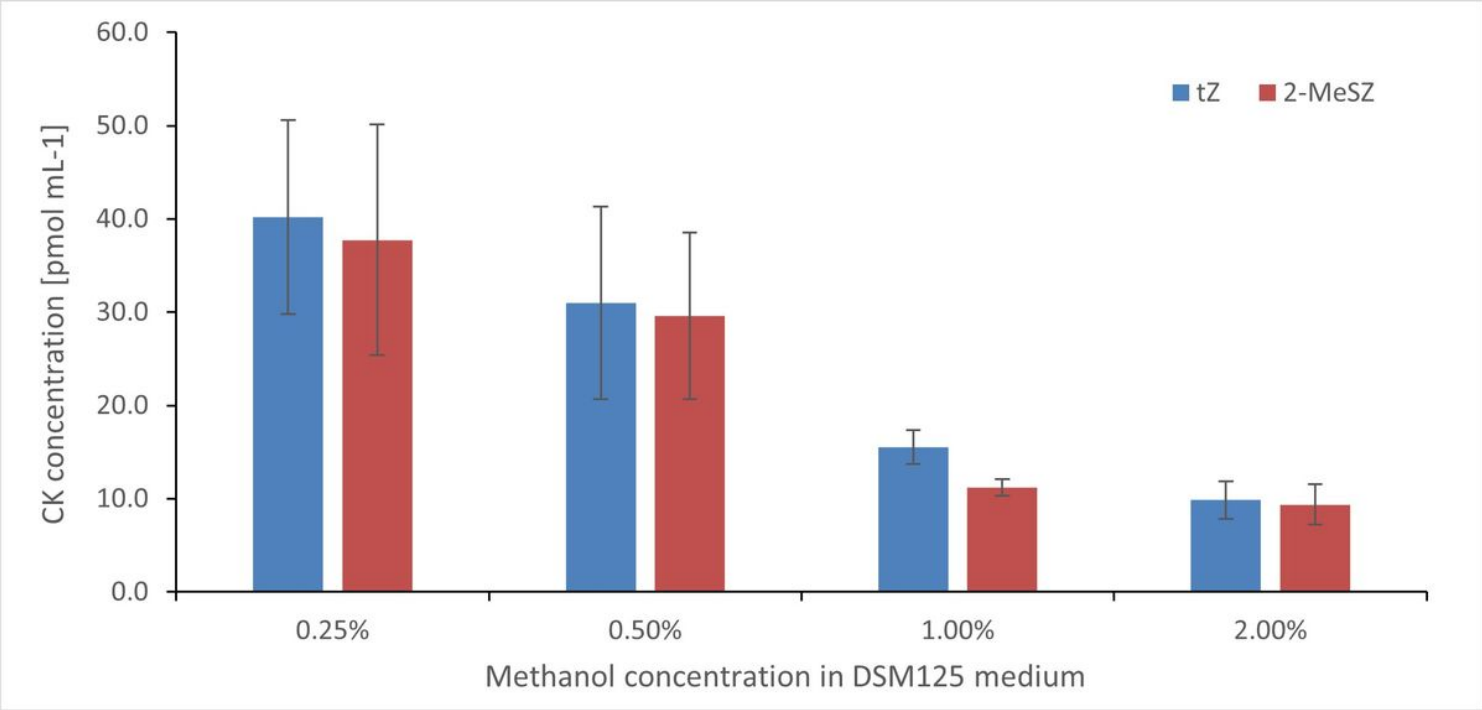

Figure 4

Cytokinin (CK) concentration (pmol mL-1) in the cell-free supernatants of Methylobacterium oryzae - LMG23582(T), cultured under different concentrations of methanol (0.25-2.00%) in the DSM125 growth medium. Cytokinin production was tested in the early stationary phase. Values are means ± SE of 3 replicates. (n=3).

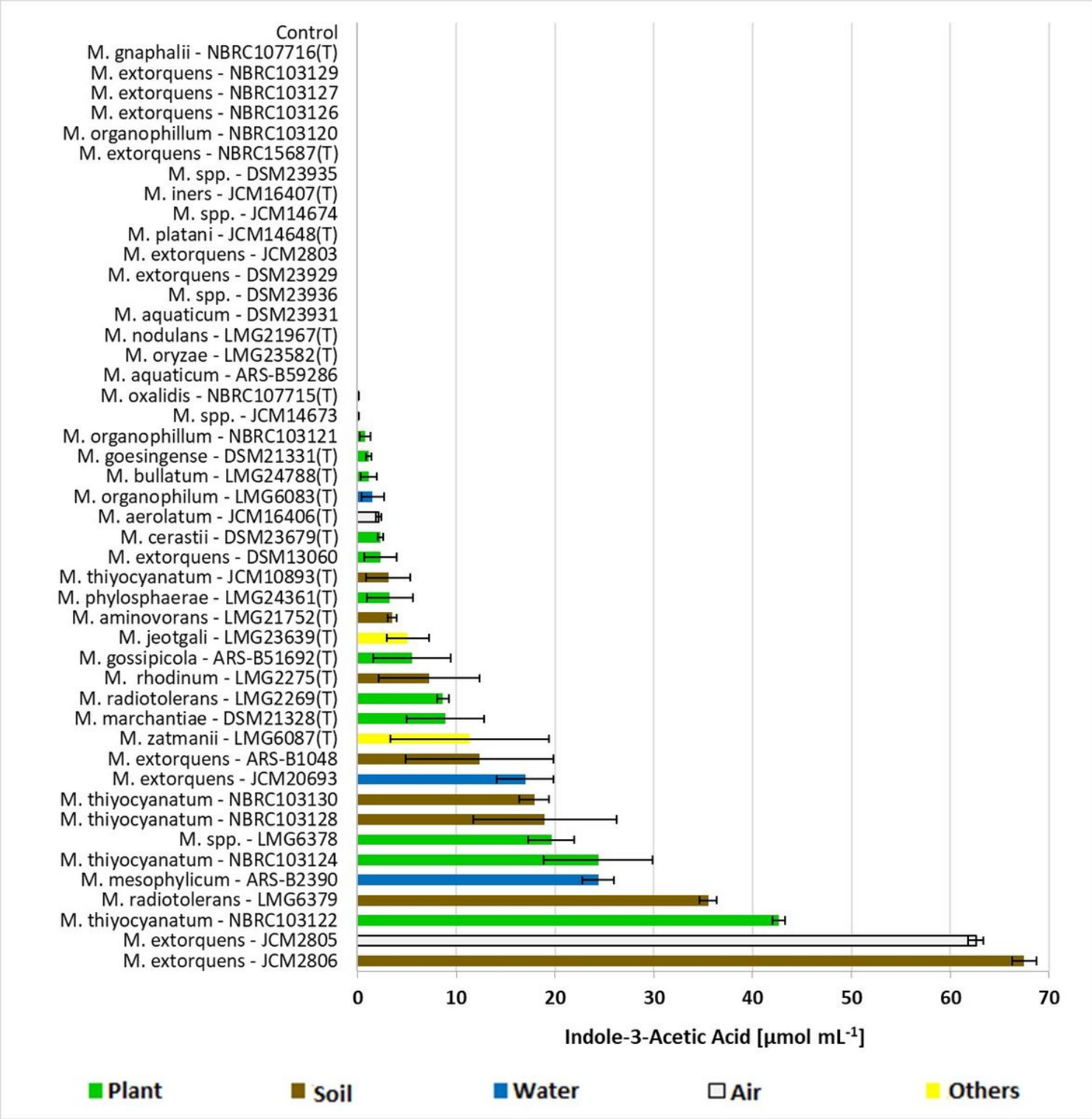

Figure 5

Total concentration of Indole-3-Acetic Acid (IAA (µg mL-1) detected spectrophotometrically by a colorimetric method in cell-free supernatants of 46 Methylobacterium strains. Strains were cultured in vitro in R2 broth supplemented with 2.5 mM L-tryptophan. Indole-3-Acetic Acid concentration was measured in the early stationary phase. Values are means ± SE of 3 replicates.

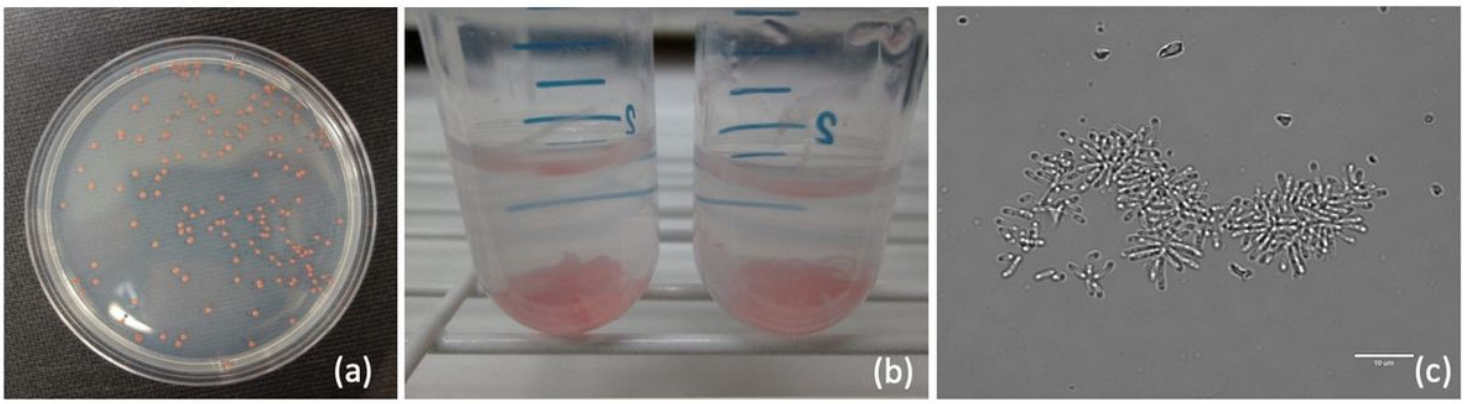

Figure 6

Methylobacterium growth on DSM125 agar plate (a) and in DSM125 liquid medium (b). Microscopic image of aggregate formation by Methylobacterium cells in liquid cultures (c).

Supplementary Files

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