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EXPERIMENTAL ARTICLES

Aerobic Methylobacteria Are Capable of Synthesizing Auxins

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Abstract—Obligately and facultatively methylotrophic bacteria with different pathways of C1 metabolism were found to be able to produce auxins, particularly indole-3-acetic acid (IAA), in amounts of 3–100 μg/ml. Indole-3-pyruvic acid and indole-3-acetamide were detected only in methylobacteria with the serine pathway of C1 metabolism (*Methylobacterium mesophilicum* and *Aminobacter aminovorans*). The production of auxins by methylobacteria was stimulated by the addition of L-tryptophan to the growth medium and was inhibited by ammonium ions. The methylobacteria under study lacked tryptophan decarboxylase and tryptophan side-chain oxidase. At the same time, they were found to contain several aminotransferases. IAA is presumably synthesized by methylobacteria through indole-3-pyruvic acid.

Key words: aerobic methylotrophic bacteria, *Methylobacterium*, *Aminobacter*, *Methylovorus*, *Paracoccus*, auxins, indole-3-acetic acid.

Aerobic methylotrophic bacteria capable of utilizing oxidized and substituted methane derivatives as sources of carbon and energy are known as methylobacteria. They are widely found in nature and are often associated with plants, where they occur on seeds and in the plant phyllosphere and rhizosphere. Some authors explain the close association of aerobic methylobacteria with plants by the functioning of the so-called methanol cycle, in which the methanol synthesized and excreted by plants is used by methylobacteria as a source of carbon and energy [1]. However, other authors believe that the relationship between methylobacteria and host plants is symbiotic in nature. Earlier, the pink-pigmented facultative methylotrophs of the genus *Methylobacterium* were found to synthesize cytokinins [2]. Using the polymerase chain reaction (PCR) technique, we revealed the cytokinin biosynthesis genes in representatives of different methylobacterial genera [3] and showed the formation of zeatin and/or zeatin riboside in *Methylobacterium mesophilicum* and the obligate methylotroph *Methylovorus mays* [4]. The fact that methylobacteria promote seed germination and the growth of seedlings [1] suggests that these bacteria may synthesize not only cytokinins, but also other phytohormones, for instance, auxins. To the best of our knowledge, there is as yet no experimental evidence that methylobacteria are able to synthesize such phytohormones.

The aim of the present work was to study the ability of aerobic methylobacteria with different pathways of C1 metabolism to synthesize auxins.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. In this study, we used three facultatively methylotrophic strains (the pink-pigmented *Methylobacterium mesophilicum* VKM B-2143 (= JCM 2829 and DSM 1708) and the colorless *Aminobacter aminovorans* ATCC 23314 and *Paracoccus kondratievae* GB VKM B-2222) and the obligately methylotrophic colorless *Methylovorus mays* strain VKM B-2221. The strains were grown in a medium containing (g/l) KH_2PO_4, 2; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 0.025; and $FeSO_4 \cdot 7H_2O$, 0.002, (pH 7.2). The growth media were supplemented with 0.5 vol % CH_3OH as the source of carbon and energy, except for the growth medium of *A. aminovorans*, which was supplemented with 0.3 wt % CH_3NH_2. In some experiments, the growth media were supplemented with 1 mM L-tryptophan or 1 mM tryptamine (Fluka, Switzerland) as auxin precursors. The effect of nitrogen sources on auxin biosynthesis was studied by adding 1 g/l KNO_3 or CH_3NH_2 to the growth medium instead of $(NH_4)_2SO_4$.

Extraction and analysis of indole compounds. The amount of indole compounds in the culture liquid of methylobacteria was determined in the late logarithmic phase after removing cells by centrifugation at 10000 *g* for 30 min. Indole compounds in the culture liquid were determined quantitatively as follows [5]. An aliquot of the culture liquid (1 volume) was mixed with 0.5 volume of the Salkowski reagent (0.05 M $FeCl_3$ in 35% $HClO_4$) and after 1 h of incubation, the optical density of the mixture was measured at 540 nm with a Specol-221 spectrophotometer (Germany). The calibration curve was constructed from the data that were obtained using indole-3-acetic acid solutions of

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known concentrations. Qualitative reactions for the presence of indoles in the culture liquid were run using the Ehrlich, Kovacs, and van Urk reagents [6].

Auxins were extracted, with an equal volume of ethylacetate, from the culture liquid acidified to pH 2.5–3.0 with HCl. The extracts were dried, the residues were dissolved in 80% ethanol [7], and the auxins present in these solutions were separated either by thin-layer chromatography (TLC) on DC Alufolien Kieselgel 60 F-254 plates (Merck, Germany) in a chloroform–ethylacetate–acetic acid (8 : 2 : 1) solvent system or by high-performance liquid chromatography (HPLC) on a Separon Six C18 (5 μm) column (Laboratorni pristroje, Czech Republic). The auxins were eluted with 30% methanol (acidified with 3-fluoroacetic acid to pH 3.0) at a flow rate of 0.5 ml/min and detected at 280 nm using an OE-308/1 UV detector (Hungary). The column was calibrated using IAA, indole-3-lactic acid (ILA), indole-3-pyruvic acid (IPyA), indole-3-acetamide, indole-3-acetaldehyde, and indole-3-methanol purchased from Sigma (United States).

Aminotransferase enzyme assay. Bacteria grown in a medium with KNO_3 in the presence of 10 mM L-tryptophan were harvested by centrifugation at 10000 *g* for 30 min and resuspended in 20 mM potassium phosphate buffer (pH 8.0) containing 10 mM EDTA, 10% glycerol, 0.1 mM dithiothreitol, and 20 μM pyridoxal phosphate. The cell suspension was sonicated at 4°C for 2 min in 30-s bursts using a 150-W MSE disintegrator (United Kingdom) operated at 20 kHz. The debris was removed by centrifugation at 15000 *g* for 1 h and the cell-free extract was analyzed by native electrophoresis in a 5–20% gradient PAA gel slab at 4°C. After electrophoresis, the gel slab was incubated at 37°C for 2 h in 50 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 6.8 mM α-ketoglutarate, 10 mM tryptophan, 3 mM NAD^+, 0.7 mM *p*-nitrotetrazolium blue, 0.13 mM phenazine methosulfate, 0.7 mM pyridoxal phosphate, and 6 U glutamate dehydrogenase.

Assay of tryptophan side-chain oxidase (TSO). Methylobacteria were grown on agar plates for 48 h (obligate methylotrophs) or 70 h (facultative methylotrophs), and then the plates were overlaid with a thin layer of a 1.2% agar medium containing 0.5 M glycine–HCl buffer (pH 3.0), 10 mM tryptophan, and 0.5% sodium dodecyl sulfate. After 12 h of incubation at room temperature, the TSO-positive colonies turned dark, whereas the TSO-negative colonies remained white [8].

Tryptophan decarboxylase assay. Methylobacteria grown in a medium with KNO_3 as the nitrogen source in the presence of 10 mM L-tryptophan were harvested by centrifugation, washed twice with sodium phosphate buffer (pH 7.6), and resuspended in 1 ml of this buffer. The cells were disrupted by ultrasound as described above. The homogenate was centrifuged at 15000 *g* for 1 h. An aliquot (0.3 ml) of the supernatant was mixed with 4.7 ml of 10 mM (600 mg/ml) tryptophan and the reaction mixture was incubated at 30°C for 16 h. Then, the pH of the mixture was adjusted to 8.0 with 0.5 M Tris–HCl buffer (pH 8.8), and the products of L-tryptophan transformation were extracted with butanol. The control butanol extract was prepared from the unincubated part of the supernatant. The butanol extracts were dried, the residues were dissolved in methanol, and the methanol solutions were analyzed by TLC on Silufol F-254 plates (Czech Republic) in an *n*-butanol–acetic acid–water (8 : 2 : 2) mixture. The activity of tryptophan decarboxylase was evaluated by the formation of tryptamine, which was determined with the ninhydrin reagent (0.6% ninhydrin in a mixture of 93 ml of *n*-butanol and 7 ml of ice acetic acid).

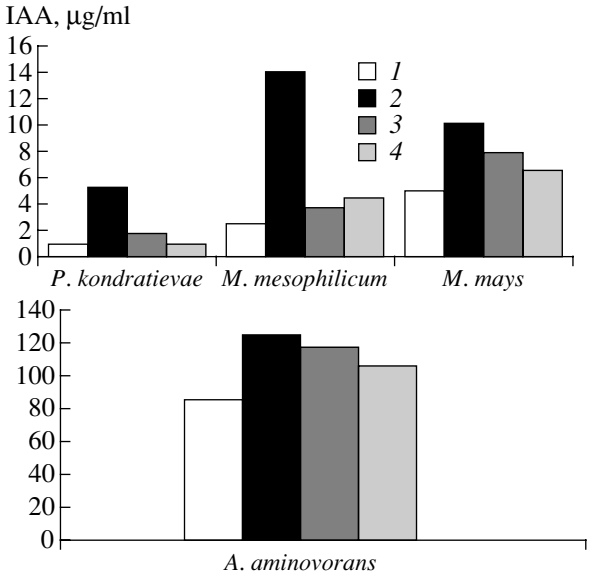

Fig. 1. IAA content in methylobacteria grown in the presence of 1 mM L-tryptophan and (*1*) $(NH_4)_2SO_4$ (pH 7.2), (*2*) KNO_3 (pH 7.2), (*3*) $(NH_4)_2SO_4$ (pH 8.2), or (*4*) CH_3NH_2 (pH 7.2).

RESULTS

Our attempts to reveal indole compounds in the culture liquids of the methylobacteria under study gave the following results. The Ehrlich and Kovacs reagents, which are usually recommended for indole detection [9], allowed us to detect indole compounds only in one methylobacterium, *A. aminovorans.* At the same time, analysis with the Salkowski and van Urk reagents revealed the formation of indoles, including IAA, by all of the methylobacteria studied. The quantitative analysis with the Salkowski reagent of the culture liquids of methylobacteria grown in a nitrate-containing medium with L-tryptophan showed that the amount of synthesized IAA varied from 5–14 μg/ml in *M. mesophilicum, M. mays,* and *P. kondratievae* to 100–120 μg/ml in *A. aminovorans* (Fig. 1). The presence of L-tryptophan in the growth medium led to the formation of a brick-red pigment. It should be noted that Paris and

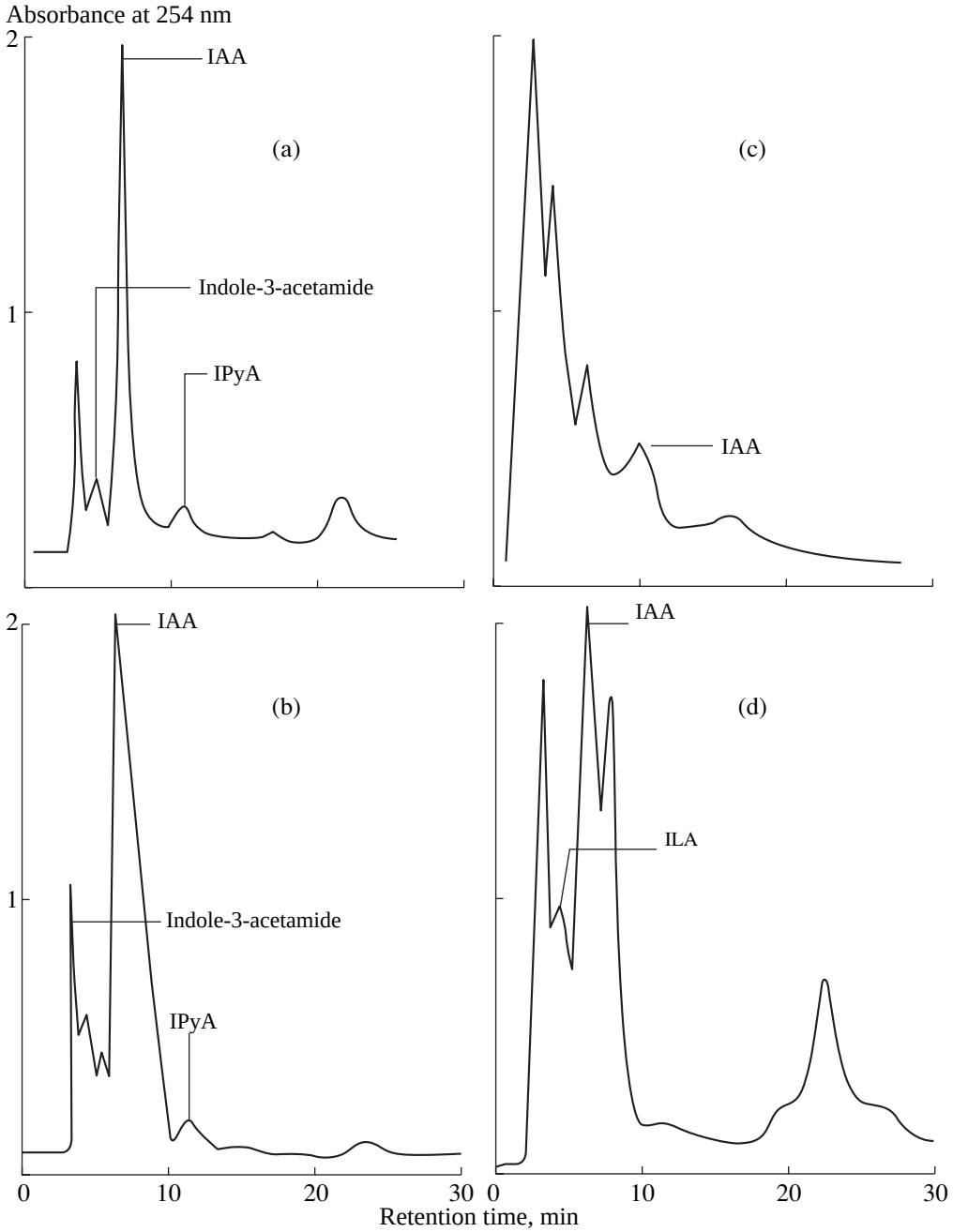

Fig. 2. The reversed-phase chromatography of auxins from the culture liquid of (a) *A. aminovorans*, (b) *M. mesophilicum*, (c) *P. kondratievae*, and (d) *M. mays* on a Separon Six C18 (5 μm) column (Laboratorni pristroje, Czech Republic). Auxins were eluted with 30% methanol, acidified with 3-fluoroacetic acid to pH 2–3 at a flow rate of 0.5 ml/min, and detected at 254 nm using an OE-308/1 UV detector (Hungary).

Magasanic explained the formation of a similar pigment in a *Klebsiella aerogenes* culture by the nonenzymatic polymerization of indole-3-pyruvate [10].

Tryptophan, which is a precursor of IAA in microorganisms and plants, frequently occurs in plant exudates. According to the observations of Schneider and Wightman [11], L-tryptophan stimulates the synthesis of auxins in certain bacteria, as it stimulated auxin synthesis in the present study. The rate of auxin synthesis strongly depended on the species and the composition of its growth medium. For instance, the obligate methylotroph *M. mays*, which is unable to grow on polycarbon substrates, synthesized indole compounds only in the presence of tryptophan. At the same time, the facultative methylotrophs *M. mesophilicum*, *A. aminovorans*, and *P. kondratievae* grown on peptone synthesized indoles in amounts of 2, 5, and 8 μg/ml, respectively, even in the absence of tryptophan in the growth

medium. Indole synthesis was strongly inhibited by ammonium ions: the substitution of KNO_3 for $(NH_4)_2SO_4$ in the growth medium led to a 2- to 15-fold increase in the indole synthesis rate (Fig. 1). Earlier, an analogous inhibiting effect of ammonium ions on indole synthesis was observed in bacteria of the genera *Azotobacter* [12] and *Pseudomonas* [13]. This effect is possibly due to the competition of ammonium ions with the amino groups of tryptophan, which are eliminated during the biosynthesis of indoles. Bearing in mind that some methylobacteria are able to use methylamines as nitrogen sources, we substituted CH_3NH_2 for $(NH_4)_2SO_4$ in the growth medium and found that such substitution did not significantly stimulate the synthesis of IAA (Fig. 1). The increase in the initial pH of the growth medium to 8.2 mitigated the inhibiting effect of ammonium ions, which was probably due to their conversion to free KNO_3.

As was shown by Crozier *et al.* in relation to the nitrogen-fixing soil bacterium *Azospirillum lipoferum*, the analysis of indoles in the growth medium with the Salkowski reagent may overestimate the indole content as compared to the data of radioisotopic analysis [14]. This can be due to the presence of nonpolar indole compounds in the culture liquid of certain bacteria that react with the Salkowski reagent but are not extracted by acidified organic solvents, such as ethylacetate or sulfur ether. In view of this, we compared the indole contents of the culture liquid of methylobacteria before and after its extraction with acidified ethylacetate and found that more than 85% of the indole compounds reacting with the Salkowski reagent are extracted by the acidified ethylacetate. Therefore, the use of the Salkowski reagent for the evaluation of the indole content of the culture liquid of methylobacteria is proved.

The identification of the indole compounds extracted from the culture liquid of methylobacteria by TLC and HPLC (Fig. 2) showed that the culture liquids of *M. mays* and *P. kondratievae* also contained, in addition to IAA, ILA. This suggests that these culture liquids may also contain IPyA, which is an extremely labile compound and an important intermediate of the tryptophan conversion pathway into IAA. Among the exometabolites of the serine-pathway methylobacteria *A. aminovorans* and *M. mesophilicum*, we found substances that are close to IPyA and indole-3-acetamide in their chromatographic mobilities.

The occurrence of ILA and IPyA in the culture liquids of the methylobacteria under study suggests that they synthesize auxins through IPyA, which is formed from tryptophan under the action of aromatic aminotransferases. Native PAA gel electrophoresis showed that each of the methylobacteria contained several proteins with aminotransferase activity (Fig. 3). This property is not unique, as some other microorganisms also exhibited the presence of more than one aromatic aminotransferase in their cells [15].

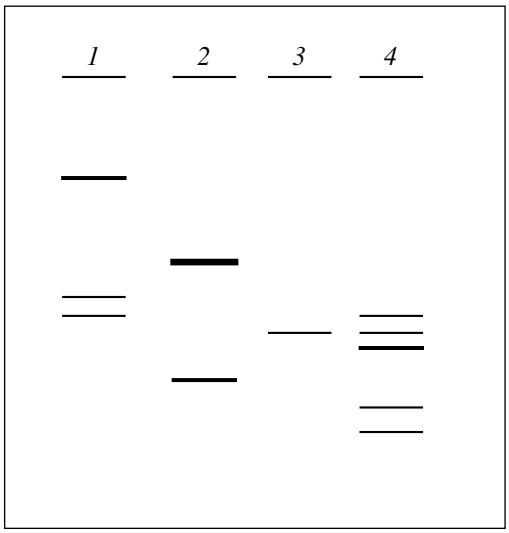

Fig. 3. Electrophoresis of proteins with aminotransferase activity from (*1*) *M. mays*, (*2*) *M. mesophilicum*, (*3*) *A. aminovorans*, and (*4*) *P. kondratievae.*

To verify the possibility that IAA is synthesized by methylobacteria through tryptamine, methylobacterial cells were incubated with 10 mM (600 mg/ml) tryptophan and the reaction products were analyzed by TLC. Such analysis failed to reveal tryptamin; therefore, tryptophan decarboxylase was probably absent as well. In accordance with these observations, tryptamine, unlike tryptophan, did not effectively stimulate the formation of IAA in the methylobacteria under study.

It is known that the conversion of tryptophan into IAA through indole-3-acetaldehyde is catalyzed by tryptophan side-chain oxidase (TSO). Our attempts to reveal this enzyme in aerobic methylobacteria by the plate technique gave negative results. Taking into account the facts that the TSO of *Pseudomonas fluorescens* is induced in the stationary growth phase (by comparison, the tryptophan aminotransferase of this bacterium is most active in the logarithmic growth phase) [8] and that the bacteria that can grow on tryptophan as the source of carbon and energy usually contain TSO, the inability of methylobacteria to grow on tryptophan and the absence of TSO activity in the stationary-phase methylobacteria grown on methanol in the presence of L-tryptophan suggest that they do lack TSO.

DISCUSSION

Many epiphytic and soil bacteria are able to synthesize auxins, primarily IAA [16, 17], due to which they influence the growth of plants either beneficially (these are bacteria of the genera *Azospirillum*, *Rhizobium*, *Pseudomonas*, and some others) or adversely (in this case, IAA acts as a factor of phytopathogenicity) [16].

Aerobic methylobacteria are a taxonomically and physiologically diverse group of microorganisms. In

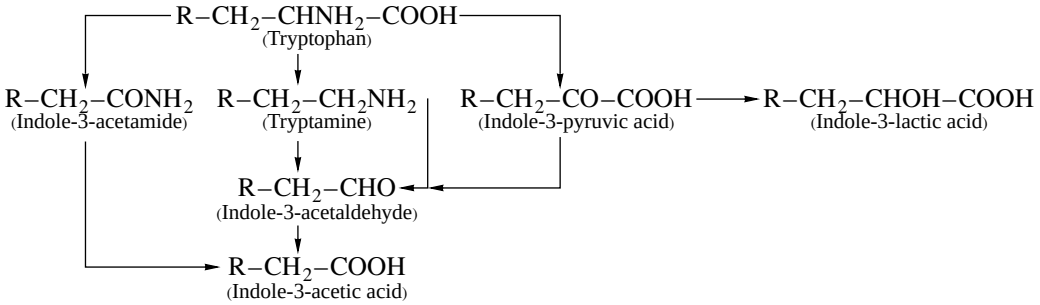

Fig. 4. IAA biosynthesis pathways in bacteria.

view of this, we chose to investigate four different species differing in their physiological and biochemical characteristics: the pink-pigmented facultative methylobacterium *M. mesophilicum* with the serine pathway of C1 metabolism, which was isolated from the phyllosphere of the perennial rye grass *Lolium perenne* L. [18]; the colorless facultative methylobacterium *A. aminovorans* with the serine pathway of C1 metabolism, which was isolated from soil [19]; the colorless obligate methylobacterium *M. mays* with the ribulose monophosphate pathway of C1 metabolism, which was isolated from the phyllosphere of the maize *Zea mays* L. [20]; and the colorless facultative methylobacterium *P. kondratievae* with the ribulose bisphosphate pathway of C1 metabolism, which was isolated from the maize rhizosphere [21].

The formation of indoles is one of the taxonomic characteristics of microorganisms. It is believed that almost all known methylotrophic and methanotrophic bacteria are unable to produce indoles. However, this belief relies on the experimental data that were obtained with the Ehrlich and Kovacs reagents [9], which are very specific for indoles but possess low sensitivity (about 50 μg/ml indoles), whereas methylobacteria usually produce lower amounts of indoles. In view of this, we used not only these two reagents for indoles but also the more sensitive Salkowski and van Urk reagents, whose sensitivity is as high as 2 μg/ml indoles. Analysis with the Ehrlich and Kovacs reagents failed to reveal indoles in the culture liquids of *M. mesophilicum*, *M. mays*, and *P. kondratievae*. In our opinion, this failure can be explained not only by the low sensitivity of the two last reagents but also by the use of the growth media that contained ammonium ions, which inhibit the synthesis of indoles from tryptophan. We may suggest that the failure of the attempts of Urakami *et al.* [20] to reveal indoles in an *A. aminovorans* culture is explained by the same reason.

The four known pathways of IAA synthesis in microorganisms involve IPyA, indole-3-acetamide, tryptamine, and indole-3-acetaldehyde with TSO (Fig. 4). The detection of IAA, ILA, IPyA, and aminotransferase activity in the culture liquids of the methylobacteria suggests that they synthesize IAA from L-tryptophan through IPyA. The absence of tryptophan decarboxylase and TSO in the methylobacteria under study confirms that they do not synthesize IAA through tryptamine.

The two-step pathway of IAA biosynthesis through indole-3-acetamide has been proved only for the phytopathogenic bacteria *Pseudomonas syringae* and *Agrobacterium tumefaciens.* The detection of indole-3-acetamide in the rhizosphere bacteria *Pseudomonas putida* and *P. fluorescens* [13] did not allow the inference to be made that the rhizosphere pseudomonads synthesize IAA via indole-3-acetamide, since the analysis of the DNA of 216 rhizosphere strains failed to reveal the necessary tryptophan monooxygenase genes. Analogously, the detection of indole-3-acetamide in the culture liquids of the methylobacteria cannot be considered to be convincing evidence that these bacteria synthesize IAA by the indole-3-acetamide pathway.

Thus, we showed that aerobic methylobacteria are able to synthesize auxins from tryptophan by the pathway that involves IPyA. Together with the earlier finding that methylobacteria are able to synthesize cytokinins, this confirms the existence of the close relationship between the methylobacteria and the host plants.

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