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SHORT COMMUNICATIONS

Production of Vitamin B_{12} in Aerobic Methylotrophic Bacteria

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It is known that methylotrophs are associated with plants and seeds [1, 2] and stimulate the growth and development of gnotobiotic plants in vitro [3–5]. Recent studies have shown that aerobic methylotrophic bacteria (methanotrophs and methylobacteria) not only play an important role in the global carbon cycle, but also act as phytosymbionts. The C_1-compounds produced by plants (methanol, halomethanes, methylsulfur compounds, and methylated amines) are involved in metabolic relations between plants and methylotrophs. In turn, the methylotrophs synthesize phytohormones (cytokinins and auxins) [6, 7], and probably other growth-stimulating factors necessary to plants, such as vitamin B_{12}.

Many eukaryotes, including higher plants [8], require this vitamin. In nature, corrinoids are usually synthesized by the *Bacteria* and *Archaea* prokaryotes [9], as well as by unicellular algae [10, 11]. There is also evidence that pink-pigmented facultative methylobacteria from the genus *Methylobacteria* produce cobalamin. The yield of this vitamin is higher on C_1- than on other C_n-substrates [12–14]. The stimulation of corrinoid synthesis by methanol may be due to activation of enzymes involved in the biosynthesis of the corrinoid ring, i.e., due to the direct or mediated effect of methanol on the expression of a cluster of *cob* genes [13]. The effect of methanol may also be related to the enzymes catalyzing the key reactions of C_1-metabolism. It remains thus far unclear whether or not the ability to produce vitamin B_{12} is widespread among aerobic methylotrophs. For this reason, the aim of this work was to measure the content of vitamin B_{12} in obligate and facultative methylotrophs with different C_1-metabolism pathways. Experiments were carried out with the type collection strains and those newly isolated from plants. The taxonomic position of the latter strains was determined from the data of 16S rRNA gene sequencing and DNA–DNA hybridization [2].

The concentration of vitamin B_{12} was measured with *Escherichia coli* strain 113-3 auxotrophic for vitamin B_{12} [15]. Measurements were performed in cell extracts, since, as a rule, this vitamin is not excreted by bacterial cells. The sensitivity of this method of vitamin determination comprised 2–4 ng vitamin B_{12} per sample. The strain *E. coli* 113-3 was maintained at 37°C on agar A, which contained the following (in g/l): tryptone, 6; L-asparagine, 0.2; K_2HPO_4 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.005; glycerol, 2; and agar, 15 (pH 6.8–7.0). After autoclaving this medium at 0.5 atm. for 30 min, it was supplemented with a sterile solution of vitamin B_{12} at a concentration of 0.1 mg/l.

For vitamin B_{12} assay, a night culture of *E. coli* 113-3 was grown in a mixture (1 : 1) of medium B and a solution containing 0.05 ng vitamin B_{12}/ml. Cultivation was performed at 37°C for 20 h. Vitamin B_{12}-free B medium contained the following (in g/l): KH_2PO_4, 7; K_2HPO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1; NaCl, 0.5; sodium citrate, 0.5; $NaNO_2$, 0.01; and glucose, 2 (pH 6.8–7.0). The medium was autoclaved at 0.5 atm. for 30 min. The inoculum (0.1 ml) was mixed with 10 ml of 0.9% NaCl and then 0.1 ml of this mixture was added to 5 ml of the aforementioned mixture (1 : 1) of medium B and vitamin B_{12}-containing solution. After incubation for 20 h, the optical density of the cell suspension was measured at 520 nm relative to the optical density of the cell suspension incubated under the same conditions in the mixture (1 : 1) of medium B and water. The amount of vitamin B_{12} in samples was determined using a calibration curve constructed with a standard solution containing 0.1 mg/ml vitamin B_{12}. Cell extracts for vitamin B_{12} measurements were prepared as follows: an aliquot of methylotrophic cells (0.5 g wet weight) harvested in the late exponential growth phase was suspended in 10 ml of the growth medium. The pH of the suspension was adjusted to 4–5 with 0.1 M HCl. The suspension was supplemented with $NaNO_2$ to a final concentration of 1 mg/ml and incubated for 15 min in a water bath at 100°C. The resultant cell extract was cooled and its pH was adjusted to 7.0. The neutralized extract was used for vitamin B_{12} assay.

It should be noted that samples may contain not only vitamin B_{12} but also methionine, which can maintain the growth of *E. coli* 113-3. For this reason, we performed an additional control analysis in which cobal-

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Content of vitamin B_{12} in aerobic methylotrophic bacteria with different pathways of C_1-metabolism

Methylotrophs	B_{12} (ng/g wet biomass)
Serine pathway	
Methylobacterium extorquens VKM B-2064^T (=NCIMB 9399^T)	41
M. extorquens AM1 VKM B-2067(=NCIMB 9133)	54
M. extorquens S_6 (isolated from soybean)	48
M. dichloromethanicum DM4 VKM B–2191^T (=DSMZ 6343^T)	50
M. mesophilicum VKM B-2143^T (=JCM 2829^T)	590
Methylobacterium sp. G-10 (cells)	800
Methylobacterium sp. G-10 (culture liquid, ng/l)	6
Methylosinus trichosporium OB3b VKM B-2117^T (= ATCC35070^T)	16
Ribulose monophosphate (RuBP) pathway	
Methylophilus methylotrophus VKM B-1623^T (=NCIMB 10515^T)	7
Methylobacillus fructoseoxidans 34 VKM B-1609	7
Methylovorus mays C VKM B-221^T (=NCIMB 13992^T)	10
Methylomonas methanica S_1 VKM B-2110^T	37
Methylomicrobium alcaliphilum 20Z VKM B-2133^T	30
Ribulose bisphosphate (RuBP) pathways	
Schlegelia plantiphila S_1 (isolated from lilac buds)	10
S. plantiphila S_2 (isolated from linden buds)	15
S. plantiphila S_4 (isolated from blue spruce needles)	14
Paracoccus sp. S_5 (isolated from linden buds)	117

amins were preliminarily decomposed. Namely, 1.5 ml of cell extract was mixed with 5 ml of 0.2 M NaOH and the mixture was incubated for 20 min in the water bath at 100°C to decompose vitamin B_{12}. Then the extracts were cooled and neutralized with 1 M HCl. These extracts were used as the controls for methionine. The mixture (1 : 1) of medium B with water served as the negative control.

As is evident from the table, facultative methylotrophs (methylobacteria with the serine and ribulose bisphosphate (RuBP) pathways of C_1-metabolism) and obligate methylotrophs (methanotrophs and methylobacteria with the serine and ribulose monophosphate (RuMP) pathways of C_1-metabolism) can produce vitamin B_{12} in various amounts. The highest amount of vitamin B_{12} was found in the pink-pigmented facultative methylobacteria of the genus *Methylobacterium*, which is in agreement with the data available in the literature [16]. Bacteria of this genus inhabit the phyllosphere of many plants. When grown under laboratory conditions, these bacteria may excrete vitamin B_{12} into the medium, provided that it is synthesized in large amounts. A large amount of vitamin B_{12} was also found in the *Paracoccus* sp. strain S_5 isolated recently from linden buds [2]. In contrast, methylotrophic bacteria of the genus *Methylophaga* were found to be vitamin B_{12}-dependent [17].

Thus, the ability to synthesize vitamin B_{12} was found not only in bacteria of the genus *Methylobacterium*, but also in the methanotrophs *Methylosinus trichosporium* with the serine pathway, *Methylomonas methanica* and *Methylomicrobium alcaliphilum* with the RuMP pathway, restricted facultative methylobacteria of the genera *Methylovorus*, *Methylophilus*, and *Methylobacillus* with the RuMP pathway, as well as in methylobacteria of the genera *Paracoccus* and *Schlegelia* with the RuBP pathway. Consequently, vitamin B_{12} is produced by almost all studied obligate and facultative aerobic methylotrophic bacteria with different pathways of C_1-metabolism. The exception is moderately halophilic methylobacteria of the genus *Methylophaga* with the RuMP pathway [17].

The inability of plants to synthesize vitamin B_{12}, which is necessary for isomerization and transmethylation reactions, is compensated for by plant-associated methylotrophs [8].

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