Metabolism and Physiology of Methylotrophic Microorganisms

Oleg Mosin[1] and Ignat Ignatov[2*]

1. PhD (Chemistry), Biotechnology Department, Moscow State University of Applied Biotechnology,

Talalikhina Street, 33, Moscow 109316, Russian Federation

2. DSc, Professor, Scientific Research Center of Medical Biophysics (SRCMB),

N. Kopernik Street, 32, Sofia 1111, Bulgaria

* E-mail of the corresponding author: mbioph@dir..bg

Abstract

Methylotrophs – aerobic chemoheterotrophic microorganisms submitted by cocci and bacilli mobile forms, are inhabitants of reservoirs and soils of various type, where there are going on various processes of decomposition of organic substances with formation of the one-carbon C_1-compounds and some C_2- and C_3-compounds, capable to be assimilated by methylotrophs. These microorganisms assimilating carbon on ribuloso-5-monophospate and serine pathways, are allocated from soil ground, the sewage containing decomposing vegetative remainss, from ruminant paunch and other sources. Methylotrophic bacteria recently draw the increasing attention of biotechnology as feasible sources of natural biologically active compounds – fodder fibers and irreplaceable amino acids, carotenoid pigments, lipids and policarbohydrates. For preparation of these compounds are used genetically marked strains of methylotrophic bacteria, obtained *via* genetic engineering approaches and selection. Recently developed gene-engineering methods of manipulation with the methylotrophic genom allow create on the basis of microbic DNA of methylotrophs expression vectors of eukaryotic proteins for medical and veterinary purposes, as human insulins. In this review article there are submitted data including the results of the authors' own research on evolution of methylotrophic bacteria, the metabolism and their biotechnological usage.

Key words: methylotrophic microorganisms, evolution, metabolism

Introduction

Methylotrophs – is a taxonomic heterogeneous group of microorganisms presented by chemoheterotrophic obligate and facultative methylotrophic bacteria and yeasts capable of assimilating carbon *via* ribulose-5-monophosphate and serine pathways of assimilation of more reduced than CO_2-carbon C_1-compounds – formaldehyde (HCOH), formic acid (HCOOH), and compounds containing either a methyl group (CH_3) or two or more methyl groups that are not directly connected with each other, as dimethyl ether $CH_3–O–CH_3$ (Gal'chenko *at al*., 1986). In nature this class of compounds is most widely occurred in natural gas, methane (CH_4), which is found in deposits of coal, oil and synthesized in large amounts by methane-forming bacteria under anaerobic conditions. The decay of pectins and other natural organic substances containing methyl esters producing methanol (CH_3OH) and ethanol (CH_3CH_2OH), which is also a substrate for growth of methylotrophic bacteria. The tissues of plants and animals contain other substrates for methylotrophic bacteria – methyl formate (CH_3COOH), methylamines (CH_3NH_2), dimethylamines ($CH_3)_2NH$, trimethylamines ($CH_3)_3N$) and their oxides.

Assimilation of C_1- compounds by microbial cell is almost always paired with breathing and, therefore is realized by strict aerobes. The only exception is the assimilation of methanol by methane-producing bacteria under anaerobic conditions. The ability to oxidize methane belongs mostly to prokaryotes. Among anaerobes such ability has methane-producing archaea, sulfate-reducing eubacteria, and some chemotrophic and phototrophic eubacteria. Methanol can also be used as a substrate for some methylotrophic yeasts.

Methylotrophs family comprises obligate and facultative aerobic eubacteria, which possess the ability to use one-carbon compounds as a source of carbon and energy. Further enumeration of such microorganisms is wide. They are represented by various Gram-positive and Gram-negative forms – representatives of the families of *Pseudomonas*, *Bacillus*, *Hyphomicrobium*, *Protaminobacter*, *Arthrobacter*, *Nocardia* and others (Romanovskaya & el Said, 1988). At present time methylotrophs are found among representatives of the following taxonomic genera: *Acidomonas, Afipia, Albibacter, Aminobacter, Amycolatopsis, Ancylobacter, Angulomicrobium, Arthrobacter, Bacillus, Burkholderia, Flavobacteria, Granulibacter, Hansschlegelia, Hyphomicrobium, Methylarcula, Methylibium, Methylobacillus, Methylobacterium, Methylohalomonas, Methyloligella,*

Methylonatrum, Methylophaga, Methylophilus, Methylopila, Methylorhabdus, Methylorosula, Methylotenera, Methyloversatilis, Methylovirgula, Methylovorus, Mycobacterium, Paracoccus, Pseudomonas, Xanthobacter.

True methylotrophic bacteria belong to the family of *Methylococcaceae*, including genera of *Methylococcus*, represented by vegetative cells resembling cocci and coccobacilli (0.7–1.5×1.0–1.5 μm) and *Methylomonas*, submitted by monads and rod-shaped cells (0.5–1.0×0.7–2.0 μm). The main taxonomic character while allocating this family is the morphological characteristics of the cells and the ability to use methane as a sole source of carbon and energy under aerobic conditions (Trocenko, 1987). Methylotrophs, assigned to the family of *Methylococcaceae*, are gram-negative eubacteria with different morphology and cell's size, which form moving or still forms. Some strains of this taxonomic family are capable to form cysts.

Interest for the study of methylotrophs is associated not only with the characteristics of their metabolism, but also with the prospects of their practical use in biotechnology as producers of high-grade feed protein and essential amino acids, as leucine and phenylalanine (Mosin *at al*., 2014), and their isotopically labeled analogues as well (Karnaukhova *et al*., 1993). The digestibility of biomass of methylotrophic bacteria amounts 85–98 %, while the productivity measured by a conversion of methanol makes up 37.6–67.5 % (Mosin *et al*., 2013) (Table 1). As we have shown, owing to strong growth in minimal salt media with methanol, high yields of biomass and bioconversion of methanol into the components of the cellular biomass (with conversion efficiency of 15.5–17.3 dry biomass per 1 g of consumed substrate) methylotrophic bacteria are considered as a cheap source of deuterated protein and amino acids (Mosin & Ignatov, 2013). The traditional approach for this is the growth of methylotrophic bacteria on minimal growth M9-media with 2 % (v/v) $C^2H_3O^2H$ and 98 % (v/v) ^2H_2O (Table 2). The profitability for obtaining the microbial protein is determined for methylotrophs, mainly by the cost of such inexpensive and available substrate, as is methanol.

Table 1: Growth parameters of different methylotrophic bacteria

Bacterial strains	The molar yield of dry biomass, g/mol of methanol	The specific growth rate, h^{-1}	The level of carbon conversion of methanol, %	The quantity of consumed nitrogen,%
Ribulose-5-monophosphate pathway of carbon assimilation				
Pseudomonas C.	17.3	0,49	67.5	13.2
Pseudomonas methanolica	17.0	0.63	66.5	11.0
Methylomonas methanolica	15.7	0.52	62.0	11.7
Serine pathway of carbon assimilation				
Pseudomonas 1	12.1	0,17	47.5	11.37
Pseudomonas 135	12.1	0.14	47.5	11.48
Pseudomonas AM1	9.8	0.09	37.6	11.20
Pseudomonas M-27	13.1	0.11	51.0	9.40
Pseudomonas roseus	13.1	0.15	51.0	10.60

The industrial value has also biotransformation carried out by methylotrophs: immobilized bacteria, cell extracts and purified enzymes for C_1-oxidation compounds, especially methanol dehydrogenase, catalyzing the oxidation of organic compounds with short chain aromatic and acyclic hydrocarbons, phenols, alcohols, and heterocyclic hydrocarbons may be used for biotransformation (Gal'chenko, 1977). Biotransformation results in obtaining a product having commercial value, for example, for obtaining propylene oxide from propylene and the substrate for the synthesis of synthetic polymers. Under certain conditions of growing the methylotrophic bacteria up to 60 % of their biomas makes up of poly-β-hydroxybutyrate – a biopolymer having big commercial value as a substitute for plastics (Suzuhi & Yamahi, 1986).

The content of phospholipids – cardiolipin, phosphatidylcholine and phosphatidylethanolamine in cell membranes of methylotrophic bacteria reaches 8–10 % (w/w) by weight of dry biomass, which makes it possible to use methylotrophic bacteria as inexpensive sources of phospholipids for the cosmetics industry, medicine and diagnostic purposes (Andreev, 1979). Furthermore, some methylotrophs are sources of cytochrome C that allows to replace the precious drug used in cardiology – cytochrome C, the source of which is the mammalian cardiac muscle (Carver & Jones, 1983).

Methylotrophs can also serve as a basis for creating genetically engineered producer strains of eukaryotic proteins for medical and veterinary usage. Thus the levels of expression of some eukaryotic genes, e.g., interferon α_1 and α_f in methylotrophic cells are higher than those ones in *E. coli* (Chistoserdov & Eremashvili, 1987).

Table 2: Amino acid composition of the protein hydrolyzate of facultative methylotrophic bacteria *Brevibacterium methylicum 5652* obtained from maximally deuterated M9-medium with 2 % (v/v) $C^2H_3O^2H$ and 98 % (v/v) ^2H_2O and levels deuteration molecules

Amino acid	Yield, % of dry weight of 1 kg of biomass of		Number of deuterium atoms incorporated into the carbon skeleton of the molecule *	The level of deuteration of molecules, % of the total number of hydrogen atoms **
	Protonated sample (control)	The sample obtained in 98 % (v/v) ^2H_2O		
Gly	8.03	9.69	2	90.0
Ala	12.95	13.98	4	97.5
Val	3.54	3.74	4	50.0
Leu	8.62	7.33	5	49.0
Ileu	4.14	3.64	5	49.0
Phe	3.88	3.94	8	95.0
Tyr	1.56	1.83	7	92.8
Ser	4.18	4.90	3	86.6
Thr	4.81	5.51	ND	ND
Met	4.94	2.25	ND	ND
Asp	7.88	9.59	2	66.6
Glu	11.68	10.38	4	70.0
Lys	4.34	3.98	5	58.9
Arg	4.63	5.28	ND	ND
His	3.43	3.73	ND	ND

Notes:

* When calculating the level of deuteration protons (deuterons) at COOH- and NH_2-groups of amino acid molecules were not considered due to their easy dissociation and isotopic ($^1H–^2H$) exchange in $H_2O/^2H_2O$.

** ND – no data.

In addition, methylotrophs are capable of synthesizing the various natural pigments (melanins, carotenoids, prodiginines) carrying out the various protective functions in the cell (Feophilova, 1974). Pigments of methylotrophic bacteria are of interest in terms of their practical use: carotenoids are used as pro-vitamins as part of feed additives and natural food colors. Melanogenny methylotrophs can be used to produce dihydroxy-phenylalanine, anticancer, radioprotective and humic substances. Currently, interest for the using of methylotrophic bacteria and components of their cell biomass in biotechnology is increasing due to the development of new technologies for chemical synthesis of methanol.

The purpose of this review article is to examine the data on the evolution of methylotrophic bacteria, the metabolism and their biotechnological application.

Results and discussion

Obligate methylotrophs

On the ability to utilize carbon methylotrophs are divided into two major taxonomic sub-groups of microorganisms – obligate and facultative methylotrophs (Tzygankov, 1986). Obligate methylotrophs are able to grow only on methane and C_1-compounds; of other substrates their growth can maintain only methanol and dimethyl ether. On the contrary, facultative methylotrophs are able to grow not only on methanol and methylamine, but on methane and some polycarbon C_n-compounds. Often they also grow on formic acid and on a small number of simple C_2- and C_4-compounds (see Table 3).

Table 3: Substrates obligate and facultative methylotrophs (for example, typical representatives of each group) (Tzygankov, 1986)

Substrates		Obligate methylotrophs (*Methylomonas*)	Facultative methylotrophs (*Hyphomicrobium*)
C_1-compounds	Methan (CH_4)	+	–
	Dimethyl ether (CH_3–O–CH_3)	+	–
	Methanol (CH_3OH)	+	+
	Formic acid (HCOOH)	–	+
C_2- compounds	Ethanol (C_2H_5OH)	–	+
	Acetic acid (CH_3COOH)	–	+
C_4- compounds	β-hydroxybutyric acid (CH_3CH (OH) CH_2COOH)	–	+

The first studied obligate methylotrophic bacterium of *Methylomonas methanica* – the Gram-negative rods with polar flagella, was described almost 100 years ago, and for several decades had been the only known bacterium capable of oxidizing methane (Figure 1). Further development and improvement of the methods of accumulation and isolation of methane oxidizing bacteria in selective nutrient media recently led to the discovery of a large number of the novel microorganisms, which are similar in properties, but different in structure. Today obligate methylotrophic bacteria are classified into the genera: *Methylococcus*, *Methylomonas*, *Methylosinus*, *Methylocystis*, *Methylobacillus*, *Methylophilus*, *Methylophaga*, *Methylovorus* and *Methylobacterium*.
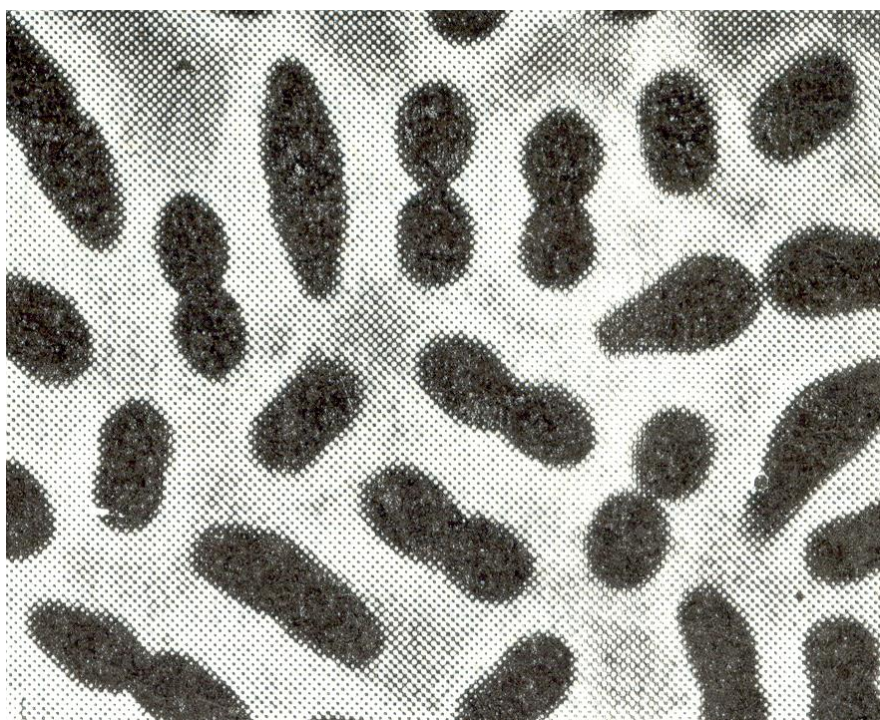

Figure 1: Electron micrograph of rod-shaped obligate methylotrophic bacterium *Methylomonas methanica* (Whittenbury *et al*., 1970)

A characteristic feature of methylotrophs is the presence in their cells the developed system of intracytoplasmic membranes, which are divided into 2 types: intra-cytoplasmic membrane of type I and intra-cytoplasmic membrane of type II (Figure 2). Intra-cytoplasmic membrane of type I is presented by stacks of tightly packed vesicular discs distributed throughout the cytoplasm, while the intra-cytoplasmic membrane of type II has the form of lamellae – a system of intra-cytoplasmic membranes, derived from the cytoplasmic membrane and kept a distinct relationship with it. These membranes have the form of individual bubbles, tubes or plates (lamellae)

arranged around the periphery of the cell cytoplasm (Davies & Whittenbury, 1970). On the topology and structure these membrane systems are reminiscent of the intra-cytoplasmic membrane of some nitrifying bacteria.

Figure 2: Electron micrographs of thin sections of cells of three obligate methylotrophs with intra-cytoplasmic membrane systems of two types: *A – Methylococcus*; membrane system of type I; *B – Methylomonas*; membrane system of type I; *C – Methylosinus*; membrane system of type II (Davies & Whittenbury, 1970)

Based on the structural features of intra-cytoplasmic membranes, all obligate methylotrophic bacteria can be divided into two main groups – rod-shaped and coccoid bacteria (Whittenbury *et al.*, 1970). Some of them form stable to drying resting cells, which in their structure resemble cysts similar to nitrogen-fixing bacteria *Azotobacter* and exospores, which are small spherical cells, spunning off from the poles of the parent cell (Figure 3).
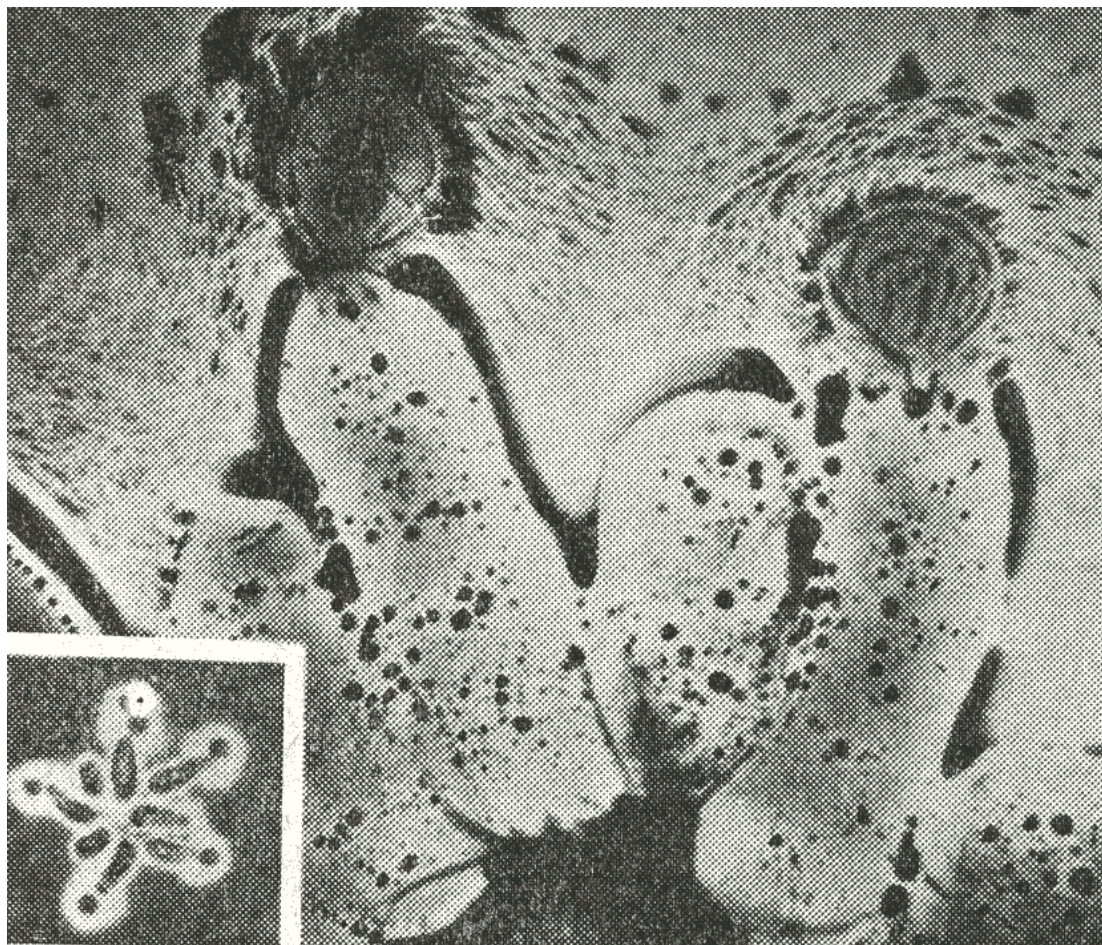
Figure 3: Electron micrograph of exospores of obligate methylotrophic bacterium *Methylosinus*. The inset at the bottom is shown the electron micrograph of budding exospores budding (Whittenbury *et al*., 1970)

The best substrates for all obligate methylotrophic bacteria are methane and methanol. The rate of bacterial growth on methanol usually is not great. Obligate methylotrophs are able to oxidize only a few organic substrates that are unable to support their growth. Such compounds include formic acid ($HCOOH$), which they oxidized to CO_2, ethylene (C_2H_4), ethyl alcohol (CH_3CH_2OH), oxidized to acetaldehyde (CH_3CHO). As a nitrogen source, these bacteria can utilize both nitrate and ammonia. However, the ammonia as being the methane oxidation inhibitor reduces the rate of bacterial growth when its concentration in growth medium is greater than 0.05 %. As a rule in nutrient media containing ammonia are formed trace amounts of nitrate. Thus, methane oxidizing bacteria are nitrifying bacteria, although scientific evidences that they can produce energy at such a small ratio of ammonium oxidation, are not numerous.

Facultative methylotrophs

To facultative methylotrophs are included some of the genera *Pseudomonas*, *Arthrobacter*, *Mycobacterium*, *Bacillus*, *Acetobacter*, *Achromobacter*, *Nocardia*, *Hyphomicrobium*, *Brevibacterium* and others. Despite the fact that obligate methylotrophs are able to grow only *via* assimilation of methanol as a sole carbon source, cumulative cultures at the utilization of the substrates are enriched with microorganisms of other types, so called facultative methylotrophs. These bacteria, unlike obligate methylotrophs are capable along with methane and methanol to assimilate as carbon sources some other one-carbon and poly-carbon compounds. The best studied of the facultative methylotrophs is the budding bacterium *Hyphomicrobium*. This bacterium is known as a powerful denitrifier, and can be isolated from enrichment cultures by incubation of microorganisms in a medium containing methanol and NH_4^+ ions under anaerobic conditions.

We isolated from aerobic enrichment cultures with methanol the leucine-dependent gram-positive rod-shaped facultative methylotrophic aerobic bacteria *Brevibacterium methylicum 5652* implementing ribulose-5-monophosphate pathway of carbon assimilation, producer of phenylalanine and other metabolically related amino acids (Mosin *et al.*, 1998). Unlike other traditional producers of *L*-phenylalanine, which do not have negative control of prephenate dehydratase (EC 4.2.1.51) or 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase (EC 2.5.1.54)*,* the uniqueness of this strain consists in that it requires *L*-leucine for growth. The initial stage of biochemical research with this strain of methylotrophic bacteria was connected with obtaining auxotrophic mutants, which in majority cases are characterized by the limited spectrum of mutant phenotypes and, besides that the high level of reversions. The initial *L*-leucine dependent strain *B. methylicum*, producer of *L*-phenylalanine was obtained *via* selection at previous stage of research after processing of parental strain by nitrozoguanidin. Screening for resistant cell colonies was carried out by their stability to the analogue of phenylalanine – *meta*-fluoro-phenylalanine (50 µg/ml). The analogue resistant mutants allocated on selective media were able to convert methanol and accumulate up to 1 gram per 1 liter of *L*-phenylalanine into growth media, which is important for biotechnological use of this strain in the production of phenylalanine.
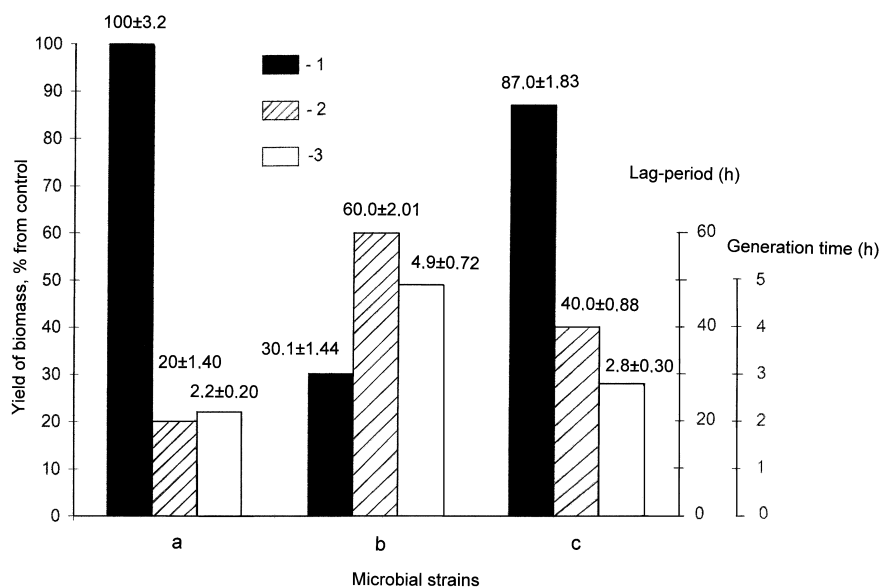

Figure 4: Yield of microbial biomass of *B. methylicum*, magnitude of lag-period and generation time in various experimental conditions: initial strain on protonated M9 medium (control) with water and methanol (*a*); initial strain on maximally deuterated M9 medium (*b*); adapted to deuterium strain on maximally deuterated M9 medium (*c*): 1 – yield of biomass, % from the control; 2 – duration of lag-period, h; 3 – generation time, h.

Further attempts were made to intensify the growth and biosynthetic parameters of this bacterium in order to grow on media M9 with higher concentration of deuterated substrates – $C^2H_3O^2H$ and ^2H_2O. For this, was applied deuterium enrichment technique *via* plating cell colonies on 2 % (w/v) agarose media M9 supplemented with 2 % (v/v) $C^2H_3O^2H$ with an increase in the ^2H_2O content from 0; 24.5; 49.0; 73.5 up to 98 % (v/v) ^2H_2O, combined with subsequent selection of cell colonies which were resistant to deuterium. The degree of cell survive on maximum deuterated medium was approx. 40 %. The data on the yield of biomass of initial and adapted *B. methylicum*, magnitude of lag-period and generation time on protonated and maximum deuterated M9

medium are shown in Figure 4. The yield of biomass for adapted methylotroph (*c*) was decreased approx. on 13 % in comparison with control conditions (*a*) at an increase in the time of generation up to 2,8 h and the lag-period up to 40 h (Figure 4). As is shown from these data, as compared with the adapted strain, the growth characteristics of initial strain on maximally deuterated medium were inhibited by deuterium.

Experimental conditions are given in Table 4 (expts. 1–10) relative to the control (expt. 1) on protonated medium M9 and to the adapted bacterium (expt. 10'). Various compositions of [U-^{2}H]MeOH and ^{2}H$_2$O were added to growth media M9 as hydrogen/deuterium atoms could be assimilated both from C^{2}H$_3$O^{2}H and ^{2}H$_2$O. The maximum deuterium content was under conditions (10) and (10') in which we used 98 % (v/v) ^{2}H$_2$O and 2 % (v/v) C^{2}H$_3$O^{2}H. The even numbers of experiment (Table 4, expts. 2, 4, 6, 8, 10) were chosen to investigate whether the replacement of CH$_3$OH by its deuterated analogue affected growth characteristics in presence of ^{2}H$_2$O. That caused small alterations in growth characteristics (Table 4, expts. 2, 4, 6, 8, 10) relative to experiments, where we used protonated methanol (Table 4, expts. 3, 5, 7, 9). The gradual increment in the concentration of ^{2}H$_2$O into growth medium caused the proportional increase in lag-period and yields microbial biomass in all isotopic experiments. Thus, in the control (Table 4, expt. 1), the duration of lag-period did not exceed 20,2 h, the yield of microbial biomass (wet weight) and production of phenylalanine were 200.2 and 0.95 gram per 1 liter of growth medium. The results suggested, that below 49 % (v/v) ^{2}H$_2$O (Table 4, expts. 2–4) there was a small inhibition of bacterial growth compared with the control (Table 4, expt. 1). However, above 49 % (v/v) ^{2}H$_2$O (Table 4, expts. 5–8), growth was markedly reduced, while at the upper content of ^{2}H$_2$O (Table 4, expts. 9–10) growth got 3.3-fold reduced. With increasing content of ^{2}H$_2$O in growth media there was a simultaneous increase both of lag-period and generation time. Thus, on maximally deuterated growth medium (Table 4, expt. 10) with 98 % (v/v) ^{2}H$_2$O and 2 % (v/v) C^{2}H$_3$O^{2}H, lag-period was 3 fold higher with an increased generation time to 2.2 fold as compared to protonated growth medium with protonated water and methanol which serve as control (Table 4, expt. 1). While on comparing adapted bacterium on maximally deuterated growth medium (Table 4, expt. 10') containing 98 % (v/v) ^{2}H$_2$O and 2 % (v/v) C^{2}H$_3$O^{2}H with non adapted bacterium at similar concentration showed 2.10 and 2.89 fold increase in terms of phenylalanine production and biomass yield due to deuterium enrichment technique, while, the lag phase as well as generation time also got reduced to 1,5 fold and 1.75 fold in case of adapted bacterium.

Table 4: Effect of variation in isotopic content (0–98 % ^{2}H$_2$O, v/v) present in growth medium M9 on bacterial growth of *B. methylicum* and phenylalanine production

Exp. number	Media components, % (v/v)				Lag-period (h)	Yield in terms of wet biomass (g/l)	Generation time (h)	Phenylalanine production (g/l)
	H$_2$O	^{2}H$_2$O	CH$_3$OH	C^{2}H$_3$O^{2}H				
1(control)	98.0	0	2	0	20.2 ±1.40	200.2 ±3.20	2.2 ±0.20	0.95 ±0.12
2	98.0	0	0	2	20.3 ±1.44	184.6 ±2.78	2.4 ±0.23	0.92 ±0.10
3	73.5	24.5	2	0	20.5 ±0.91	181.2 ±1.89	2.4 ±0.25	0.90 ±0.10
4	73.5	24.5	0	2	34.6 ±0.89	171.8 ±1.81	2.6 ±0.23	0.90 ±0.08
5	49.0	49.0	2	0	40.1 ±0.90	140.2 ±1.96	3.0 ±0.32	0.86 ±0.10
6	49.0	49.0	0	2	44.2 ±1.38	121.0 ±1.83	3.2 ±0.36	0.81 ±0.09
7	24.5	73.5	2	0	45.4 ±1.41	112.8 ±1.19	3.5 ±0.27	0.69 ±0.08
8	24.5	73.5	0	2	49.3 ±0.91	94.4 ±1.74	3.8 ±0.25	0.67 ±0.08
9	98.0	0	2	0	58.5 ±1.94	65.8 ±1.13	4.4 ±0.70	0.37 ±0.06
10	98.0	0	0	2	60.1 ±2.01	60.2 ±1.44	4.9 ±0.72	0.39 ±0.05
10'	98.0	0	0	2	40.2 ±0.88	174.0 ±1.83	2.8 ±0.30	0.82 ±0.08

Notes:

* The date in expts. 1–10 described the growth characteristics for non-adapted bacteria in growth media, containing 2 % (v/v) CH$_3$OH/C^{2}H$_3$O^{2}H and specified amounts (%, v/v) of ^{2}H$_2$O.

** The date in expt. 10' described the growth characteristics for bacteria adapted to maximum content of deuterium in growth medium.

***As the control used exprt. 1 where used ordinary protonated water and methanol

The adapted *B. methylicum* eventually came back to normal growth at placing over in protonated growth medium after some lag-period that proves phenotypical nature of a phenomenon of adaptation that was observed for others adapted by us strains of methylotrophic bacteria and representatives of other taxonomic groups of microorganisms (Mosin *et al*., 2012; Mosin & Ignatov, 2014a). The effect of reversion of growth in protonated/deuterated growth

media proves that adaptation to ^2H_2O is rather a phenotypical phenomenon, although it is not excluded that a certain genotype determines the manifestation of the same phenotypic trait in the growth media with different isotopic compositions. On the whole, improved growth characteristics of adapted methylotroph essentially simplify the scheme of obtaining the deutero-biomass which optimum conditions are M9 growth medium with 98 % (v/v) ^2H_2O and 2 % (v/v) $C^2H_3O^2H$ with incubation period 3–4 days at temperature 35 ^{0}C.

Literature reports clearly reveal that the transfer of deuterated cells to protonated medium M9 eventually after some lag period results in normal growth that could be due to the phenomenon of adaptation wherein phenotypic variation was observed by the strain of methylotrophic bacteria. The effect of reversion of growth in protonated/deuterated growth media proves that adaptation to ^2H_2O is a phenotypical phenomenon, although it cannot be excluded that a certain genotype determined the manifestation of the same phenotypic attribute in media with high deuterium content. The improved growth characteristics of the adapted bacterium essentially simplify the obtaining of deutero-biomass in growth medium M9 with 98 % (v/v) ^2H_2O and 2 % (v/v) $C^2H_3O^2H$.
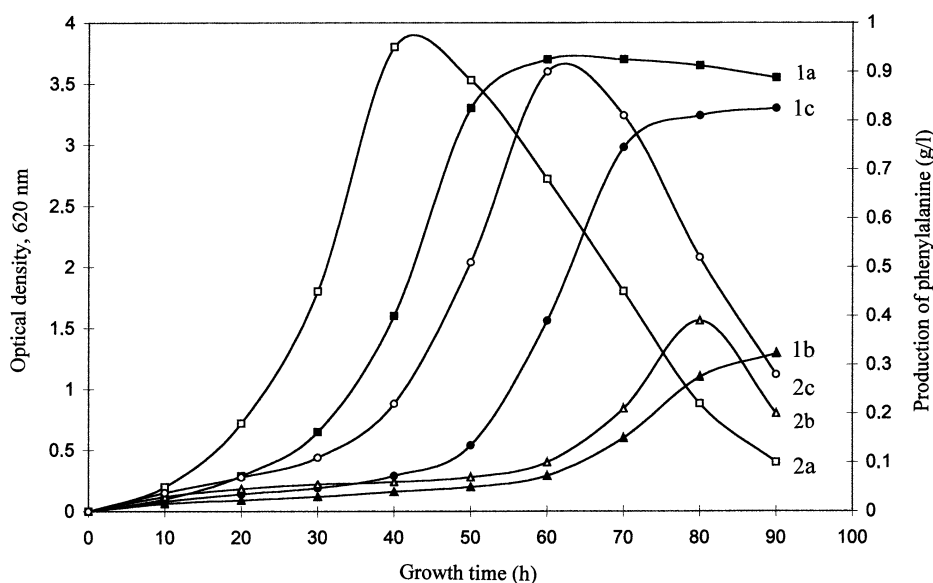

Figure 5: Growth dynamics of *B. methylicum* (1a, 2a, 3a) and production of phenylalanine (1b, 2b, 3b) on media M9 with various isotopic content: 1a, 1b – non-adapted bacterium on protonated medium (Table 1, expt. 1); 2a, 2b – non-adapted bacterium on maximally deuterated medium (Table 1, expt. 10); 3a, 3b – adapted bacterium on maximally deuterated medium (Table 1, expt. 10')

Adaptation, which conditions are shown in experiment 10' (Table 4) was observed by investigating of growth dynamics (expts. 1*a,* 1*b,* 1*c*) and accumulation of *L*-phenylalanine into growth media (expts. 2*a,* 2*b,* 2*c*) by initial (*a*) and adapted to deuterium (*c*) strain *B. methylicum* in maximum deuterated growth medium M9 (Figure 5, the control (*b*) is obtained on protonated growth medium M9). In the present study, the production of phenylalanine (Fig. 5, expts. 1b, 2b, 3b) was studied and was found to show a close linear extrapolation with respect to the time up to exponential growth dynamics (Fig. 5, expts. 1a, 2a, 3a). The level of phenylalanine production for non-adapted bacterium on maximally deuterated medium M9 was 0,39 g/liter after 80 hours of growth (Fig. 5, expt. 2b). The level of phenylalanine production by adapted bacterium under those growth conditions was 0,82 g/liter (Fig. 5, expt. 3b). Unlike to the adapted strain the growth of initial strain and production of phenylalanine in maximum deuterated growth medium were inhibited. The important feature of adapted to ^2H_2O strain *B. methylicum* was that it has kept its ability to synthesize and exogenously produce *L*-phenylalanine into growth medium. Thus, the use of adapted bacterium enabled to improve the level of phenylalanine production on maximally deuterated medium by 2,1 times with the reduction in the lag phase up to 20 h. This is an essential achievement for this strain of methylotrophic bacteria, because up till today there have not been any reports about production of phenylalanine by leucine auxotrophic methylotrophs with the NAD$^+$ dependent methanol dehydrogenase (EC 1.6.99.3) variant of the RuMP cycle of carbon assimilation. This makes this isolated strain unique for production of phenylalanine.

The general feature of phenylalanine biosynthesis in $H_2O/^2H_2O$-media was increase of its production at early exponential phase of growth when outputs of a microbial biomass were insignificant (Figure 5). In all the experiments it was observed that there was a decrease in phenylalanine accumulation in growth media at the late exponential phase of growth. Microscopic research of growing population of microorganisms showed that the character of phenylalanine accumulation in growth media did not correlate with morphological changes at various

stages of the cellular growth. Most likely that phenylalanine, accumulated in growth media, inhibited enzymes of its biosynthetic pathways, or it later may be transformed into intermediate compounds of its biosynthesis, e.g. phenylpyruvate. Phenylalanine is synthesised in cells of microorganisms from prephenic acid, which through a formation stage of phenylpiruvate turns into phenylalanine under the influence of cellular transaminases. However, phenylalanine was not the only product of biosynthesis; other metabolically related amino acids (alanine, valine, and leucine/isoleucine) were also produced and accumulated into growth media in amounts of 5–6 µmol in addition to phenylalanine.

With increasing of ^2H_2O content in growth media, the levels of deuterium enrichment in [^2H]amino acid molecules were varried propotionaly. The similar result on proportional specific increase of levels of deuterium enrichment into [^2H]phenylalanine and other metabolically related [^2H]amino acids (alanine, valine and leucine/isoleucine) was observed in all isotopic experiments where used increasing concentration ^2H_2O in growth media (Table 5). Predictably, enrichment levels of [^2H]phenylalanine related to the family of aromatic amino acids synthesised from shikimic acid and metabolically related [^2H]amino acids of pyruvic acid family – alanine, valine and leucine at identical ^2H_2O concentration in growth media are correlated among themselves. Such result is fixed in all isotope experiments with ^2H_2O (Table 5). Unlike [^2H]phenylalanine, deuterium enrichment levels in accompanying [^2H]amino acids – Ala, Val and Leu/Ile keep a stable constancy within a wide interval of ^2H_2O concentration: from 49 % (v/v) to 98 % (v/v) ^2H_2O (Table 5). Summarizing these data, it is possible to draw a conclusion on preservation of minor pathways of the metabolism connected with biosynthesis of leucine and metabolic related amino acids of pyruvic acid family – alanine and valine, which enrichment levels were in correlation within identical concentration of H_2O in growth media (phenylalanine is related to the family of aromatic amino acids synthesized from shikimic acid). Since leucine was added into growth media in protonated form, another explanation of this effect, taking into consideration the various biosynthetic pathways of Leu and Ileu (Ileu belongs to the family of aspartic acid, while Leu belongs to the pyruvic acid family), could be cell assimilation of protonated leucine from growth media. Since Leu and Ileu could not be clearly estimated by EI MS method, nothing could be said about possible biosynthesis of [^2H]isoleucine. Evidently, higher levels of deuterium enrichment can be achieved by replacement of protonated leucine on its deuterated analogue, which may be isolated from hydrolysates of deuterated biomass of this methylotrophic bacterium.

Table 5: Effect of deuterium enrichment levels (atom%) in the molecules of [^2H]amino acids excreted by *B. methylicum**

[^2H]amino acid	Concentration of ^2H_2O in growth media, % (v/v)**			
	24.5	49.0	73.5	98.0
Ala	24.0±0.70	50.0±0.89	50.0±0.83	50.0±1.13
Val	20.0±0.72	50.0±0.88	50.0±0.72	62.5±1.40
Leu/Ileu	20.0±0.90	50.0±1.38	50.0±1.37	50.0±1.25
Phe	17.0±1.13	27.5±0.88	50.0±1.12	75.0±1.40

Notes:

* At calculation of enrichment levels protons (deuterons) at COOH- and NH_2-groups of amino acids were not considered because of dissociation in H_2O (^2H_2O).

** The data on enrichment levels described bacteria grown on minimal growth media M9 containing 2 % (v/v) $C^2H_3O^2H$ and specified amounts (%, v/v) of ^2H_2O.

Metabolism of methyl compounds

The process of enzymatic oxidation of methane in the cells of methanotrophs can be represented schematically in Figure 6.

The initial stage of the oxidation of methane to methanol is catalyzed by $NAD^.H_2$-dependent methane monooxygenase – a key enzyme of aerobic methanotrophs; other enzymes are present in other members of methylotrophs. The literature describes two forms of this enzyme: associated with intra-cytoplasmic membranes and soluble methane monooxygenase (Anthony, 1986). Electron donor for the first form of the enzyme can be the reduced cytochrome (Cyt) or $NAD^.H_2$, forming as a result of the reversed electron transport; for the second form of the enzyme – only $NAD(P)^.H_2$ or compounds which are oxidized with its formation. Carbon are fixed by the cell at the stage of carbon dioxide (3) or formaldehyde formation (2) (Figure 6).

Subsequent stages of the enzymatic oxidation of methanol are catalyzed by relevant dehydrogenases – CytC dependent methanol dehydrogenase, CytC-dependent formaldehyde dehydrogenase (or its function is performed by methanol dehydrogenase) and NAD^+-dependent formate dehydrogenase, differing by the structure, the nature of electron acceptors, and other parameters.
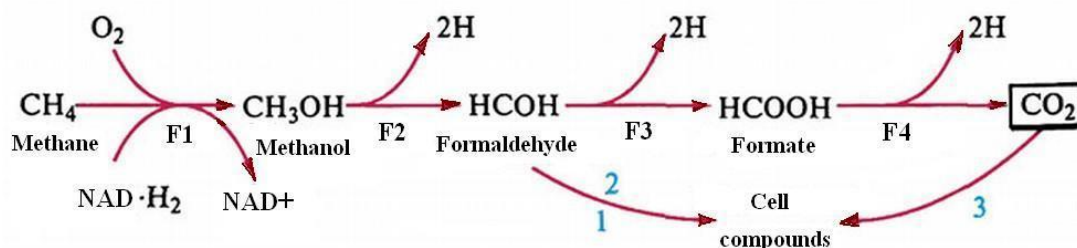
Figure 6: Diagram of the enzymatic oxidation of methane: F1 – methane monooxygenase; F2 – methanol dehydrogenase; F3 – formaldehyde dehydrogenase; F4 – formate dehydrogenase

The energy efficiency of the oxidation of C_1-compounds by relevant dehydrogenases is determined by the place of receipt of the electrons along the respiratory chain, which in composition of transporters and their localization on the membrane is similar to those ones typical for most aerobic eubacteria. In the oxidative metabolism of C_1-compounds are involved $NAD^.H^+$, flavins, quinones, cytochromes *a*, *b*, *c*. Oxidation of methanol to formaldehyde, catalyzed by methanol dehydrogenase containing as prosthetic group the residue of pyrrolo-quinoline quinone (coenzyme PQQ), is accompanied by the transfer of electrons in the mitochondrial respiratory chain at the level of cytochrome c. This process leads to the synthesis of ATP molecule (Figure 7).
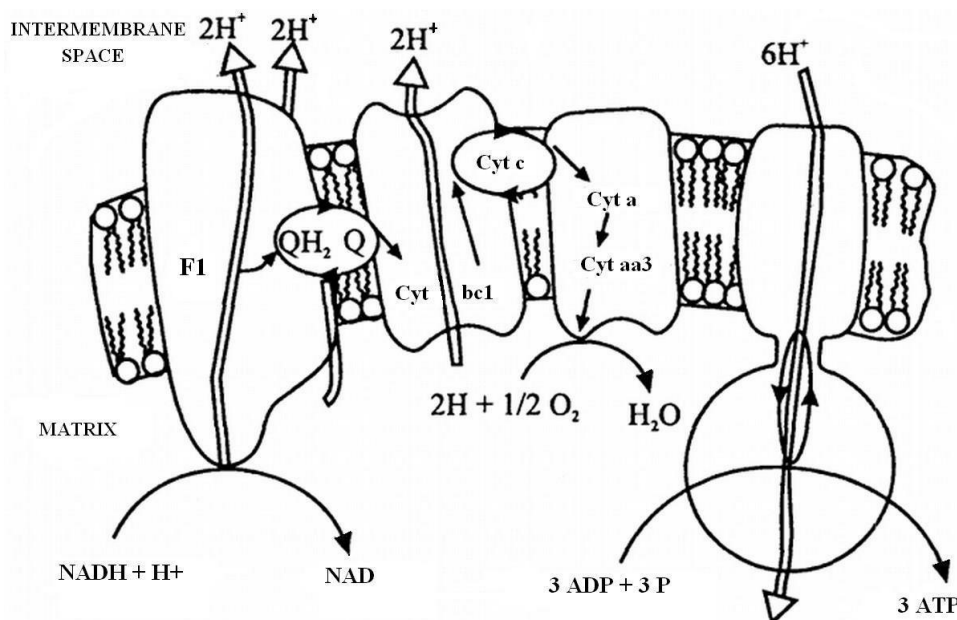

Figure 7: The respiratory electron transport chain: Cyt – cytochrome, Q – quinone, Fl – flavoprotein.

Formaldehyde in methylotrophs is a key metabolite, at which diverge the constructive and energy pathways (Quayle, 1972). Part amount of formaldehyde is converted into cell substances *via* the specific to methylotrophic bacteria assimilation cyclic pathways, while most part of formaldehyde is oxidized with NAD^+-dependent formaldehyde dehydrogenase to formate, which is further cleaved to CO_2 with using formate dehydrogenase.

The NAD^+-dependent oxidation of formaldehyde and formate suggesting that the transfer of an electron pair can be linked to the transmembrane movement of protons involving ATP. The experimental data indicate, however, for smaller outputs of ATP. However, the question on the level at which the electrons are transferred from formaldehyde and formate into the respiratory chain is not entirely clear.

Some Gram-positive methylotrophic bacteria can utilize as a substrate for their growth diethyl ether (C_2H_5–O–C_2H_5). This compound is cleaved at oxidation by oxygenation, resulting in formation of ethanol (CH_3CH_2OH) and acetaldehyde (CH_3CHO):

$$C_2H_5–O–C_2H_5 + O_2 + NAD^.H + H^+ \rightarrow CH_3CH_2OH + CH_3CHO + NAD^+ + H_2O \quad (1)$$

A similar oxidation of dimethyl ether to methane in bacteria may result in formation of methanol and formaldehyde, although the mechanism of the reaction is not completely understood:

$$CH_3-O-CH_3 + O_2 + NAD \cdot H^+ \rightarrow CH_3OH + HCHO + NAD^+ + H_2O \qquad (2)$$

In contrast to the oxidation of methanol occurring with the participation of methanol dehydrogenase, the oxidation of primary alcohols is carried out by pyridine-dependent dehydrogenases.

Experiments carried out by us on the study of the incorporation of deuterium into the components of the cellular biomass by assimilation C^2H3O^2H at the growth of methylotrophic bacteria *Methylobacillus flagelatum KT* and *Brevibacterium methylicum 5652* on the growth media with 2 % (v/v) C^2H3O^2H, showed a small amount of deuterium entering into the molecules with carbon of C^2H3O^2H (not more than 5 %) (Mosin & Ignatov, 2014b). This result is explained by dilution of the deuterium label at the expense of biochemical processes associated with the decomposition of C^2H3O^2H at its assimilation by the cell, and the isotopic exchange reaction and dissociation in aqueous media. Thus, of four deuterium atoms in the molecule of C^2H3O^2H, only one deuterium atom at the hydroxy group $-O^2H$ is most mobile and therefore readily dissociates in an aqueous medium to form C^2H3OH. The three remaining deuterium atoms in the molecule of C^2H3O^2H are entered into the cycle of enzymatic oxidation of methanol that leads to loss of deuterium label due to the formation of compounds being more oxidized than methanol. In particular, such incorporation of deuterium into the components of the cellular biomass confirms the classical scheme of the enzymatic oxidation of methanol to formaldehyde in methylotrophs cells, which then utilized by ribulose-5-monophosphate or serine pathway of carbon assimilation.

Assimilation of carbon by methylotrophic bacteria

Methylotrophs are able to form carbon in cells from C_1-compounds, as from organic substrate, as well as due to the assimilation of CO_2, formed at oxidation of C_1-compounds in the reduced oxidative pentose phosphate pathway (Calvin cycle), resulting in fixation of carbon dioxide and formation from it the hexose molecule (Trotsenko & Murrel, 2008). Experiments with using ^{13}C-labeled carbon substrates showed that the bulk of carbon in the cell was derived from oxidized substrate, instead of CO_2. The tricarboxylic acid cycle (TCA cycle) also plays no significant role in the catabolic pathways of methylotrophs, because TCA-cycle enzyme activity in the cells of methylotrophs is relatively low. In fact, the carbon source is an intermediate – formaldehyde, which in methylotrophs is a key metabolite, at which diverge structural and energy pathways leading to the two main pathways of assimilation of C_1-compounds by the cell – ribulose-5-monophosphate and serine pathways of carbon assimilation (Trotsenko *et al*., 1995). The enzymes which catalyze biochemical reactions are specific for each cycle.

The ribulose-5-monophosphate (RMP) pathway in many respects is similar to the Calvin cycle with CO_2 assimilation with the difference that in this cycle as an acceptor of CO_2 acts the pentose molecule. The key reaction of RMP cycle is the addition of formaldehyde to ribulose-5-phosphate catalyzed by hexoso phosphate synthase with forming a phosphorylated sugar – hexulose-6-phosphate, which is then isomerized to fructose-6-phosphate with participation of phospho hexulose isomerase (Figure 8, *A*). Then fructose-6-phosphate further is subjected to phosphorylation with phosphofructokinase. The resulting fructose-1,6-diphosphate is splitted into two molecules of trioses: 3-phosphoglyceraldehyde (3-PGA) and phospho-dioxyacetone, which are used for further enzymatic reactions. 3-PGA and fructose-6-phosphate is involved in a series of reactions leading to the regeneration of the acceptor of formaldehyde – ribulose-5-phosphate. These reactions are similar to those ones for the reduced oxidative pentose phosphate pathway, in which there occurs the catalyzed by the ribulose diphosphate carboxylase the acceptance by the ribulose-1,5-bisphosphate a molecule of CO_2 and subsequent hydrolytic cleavage of the resulting hexoses into 2 molecules of 3-phosphoglyceric acid (3-PG), subjected to a series of sequential enzymatic reactions leading to the formation of a molecule of glucose. However, the reduced oxidative pentose phosphate pathway is not widespread in methylotrophs and is found only in their individual representatives, which are able to grow autotrophically as well as those ones which can utilize formic acid (HCOOH) due to CO_2 assimilation. A prerequisite for the growth of methylotrophs on formic acid is the ability to synthesize the two key enzymes of the pathway – phospho ribulokinase and ribulose diphosphate carboxylase.
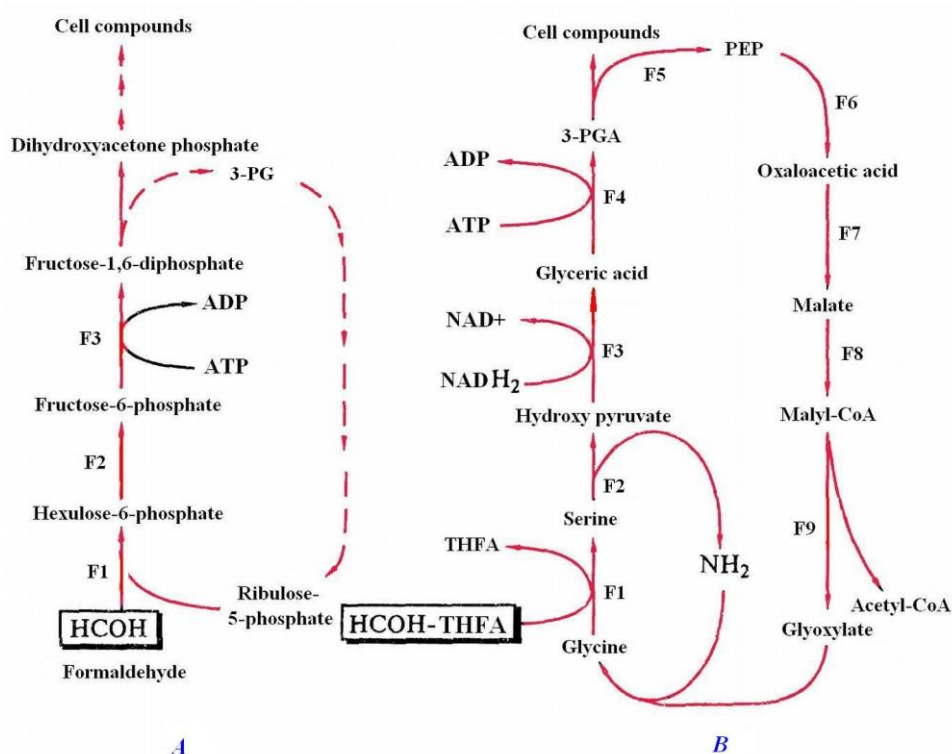
Figure 8: The pathways of assimilation of formaldehyde by methylotrophs. *A* – The ribulose-5-monophosphate pathway: F1 – hexose phosphate synthase; F2 – phospho hexulose isomerase; F3 – phosphofructokinase; F4 – fructose-biphosphate aldolase; dashed line indicates the reactions of regeneration of ribulose-5-phosphate, similar to the corresponding reduced oxidative pentose phosphate pathway. *B* – The serine pathway: F1 – serine hydroxymethyltransferase; F2 – serine glyoxylate aminotransferase; F3 – hydroxy pyruvate reductase; F4 – glycerate kinase; F5 – enolase; F6 – PEP carboxylase; F7 – malate dehydrogenase; F8 – malate tiokinaze; F9 – malyl-CoA lyase

The serine pathway differs significantly from the RMP pathway by the nature of formed intermediates and enzymes (Figure 8, *B*). The key enzyme in this pathway is serine hydroxymethyltransferase which catalyzes the formation of serine from glycine and formaldehyde presented in the form of a derivative of tetrahydrofolic acid (THFA):

$$HCOH– THFA + CH_2NH_2COOH \rightarrow CH_2OHCHNH_2COOH + THFA \qquad (3)$$

Then through a chain of consecutive reactions of transamination, the consistent recovery and phosphorylation of which leads to the formation of 3-phosphoglyceric acid (3-PGA) (Figure 8, *B*). One part of 3-PGA is used for regeneration of glycine, the primary acceptor of C_1; another portion of 3-PGA under the influence of the enzyme enolase is converted into phosphoenolpyruvic acid (PEP) and then participates in subsequent reactions. Subsequent carboxylation of PEP with PEP-carboxylase results in the synthesis of the molecule of oxaloacetic acid (OAA). This reaction is notable because at this stage into the serine cycle CO_2 is introduced. Then OAA with involving the malate dehydrogenase is converted to malate, which in turn under the influence of malil-CoA lyase splits into glyoxylic acid and acetyl-CoA. Subsequent series of reactions lead to regeneratation of glycine, and thus, the cycle is closed (Figure 8, *B*).

Investigation of the distribution of two cyclic pathways of carbon assimilation in facultative and obligate methylotrophs – RMP and serine pathways has led to the establishment of interesting features on the structure of the intra-cellular membrane (Anthony, 1982). Thus, the assimilation of formaldehyde through the RMP pathway is characteristic for methylotrophs with type I of membrane organization, and through the serine pathway – for methylotrophs with a system of intracytoplasmic membranes of type II. Another distinctive feature is that in the facultative methylotrophs the serine pathway is more common. Among the obligate methylotrophs the serine pathway functions only in those microorganisms which have membrane system of type II (*Methylosinus*, *Methylocystis*), and RMP pathway – in methylotrophs with a membrane system of type I (*Methylomonas*, *Methylobacter*, *Methylococcus*).

Evolution of methylotrophic bacteria

Since the nutritional needs of chemoautotrophs and methylotrophs are very simple, they were considered to be primitive organisms belonging perhaps to the earliest forms of life on Earth (Ignatov & Mosin, 2014a; Ignatov & Mosin, 2014b). Lately the submission of their place in the evolution of living organisms have changed. Biochemical apparatus of methylotrophic bacteria is just as complicated as that one for most of chemo-heterotrophs (Kelly, 1970). It is supposed that the first living organisms on Earth originated in anaerobic conditions, when primitive ocean was rich in organic matter formed at earlier stades of evolution. Oxygen-rich biosphere arose much later, about 2 billion years. ago. This important geochemical revolution in evolution is explained by photosynthesis. With such a character of the evolution the aerobic chemo-autotrophs and methylotrophs could appear only after oxygenic photosynthesis had been evolved. It can be assumed that chemo-autotrophs and methylotrophs could evolved from common prokaryotic microorganisms precursors that carry out photosynthesis, but lost the photosynthesis apparatus, and their electron transport chain functioning in photosynthesis, began to carry out a new feature of assimilation of C_1-compounds. Some contemporary representatives of the two major groups of prokaryotes, photosynthetic and non-photosynthetic ones, have very interesting properties. These ones include the existence of several complex characteristic of these types of systems of internal membranes; absence of a functioning of tricarboxylic acid cycle; availability of the Calvin cycle, or its analogue, the pentose phosphate cycle; localization in carboxysomes the key enzyme of the Calvin cycle (ribulose diphosphate carboxylase). Recent studies suggest the role of methylotrophs in the evolution of microorganisms. Methylotrophs play a crucial role in the circulation of methane and other C_1-compounds in the biosphere, which are maintained at a constant level, mainly due to the activity of methylotrophs.

Conclusion

Methylotrophs − is taxonomycally heterogeneous group of microorganisms presented by chemoteterotrophic obligate and facultative methylotrophic bacteria and yeasts, capable of assimilating carbon from more reduced than CO_2 C_1-compounds. Practical interest to this taxonomic group of methylotrophs is associated with the peculiarities of their metabolism and with the prospects of their practical use in biotechnology as producers of high-grade feed protein and essential amino acids, and other important natural biologically active compounds, such as pigments, carbohydrates and lipids. Digestibility of biomass of methylotrophic bacteria eukaryotes makes up 85–98 %, and the productivity, as measured by a conversion of methanol amountds 37.6–67.5 %. Due to good growth in minimal media with methanol, high yields of biomass and bioconversion level of methanol into the components of the cell biomass (with conversion efficiency 15.5–17.3 g dry biomass per 1 g of consumed substrate) methylotrophic bacteria are regarded as cheaper sources of protein and essential amino acids. The profitability for obtaining the microbial protein is determined for methylotrophs mainly by the cost of such inexpensive and available substrate, as is methanol.

References:

Andreev, L.V. (1979) *Biosynthesis and lipid metabolism in microorganisms*. − Pushchino ONTI NCBI USSR Academy of Sciences, P. 31.

Anthony, C. (1982) The biochemistry of methylotrophs. Methylotrophic microorganisms / C. Anthony. − London, New York: Academic Press, pp. 351−378.

Anthony, C. (1986) *Bacterial oxidation of methane and methanol* / in: Advances in Microbial Physiology / A.H. Rose, D.W. Tempest (eds.) − New York: Academic Press, 27: 114−210.

Carver, M.A. & Jones, C.W. (1983) The role of C-type cytochromes in the terminal respiratory chain of the methylotrophic bacterium *Methylophilus methylotrophus. Arch. Microbiol.*, 47: 275−280.

Chistoserdov, A.Y. & Eremashvili, M.P. (1987) Gene expression of human interferon α_F in obligate methylotroph *Methylobacillus flagellatum KT. Molecular Genetics, Microbiology and Virology*, 8: 36−41.

Davies, S.L. & Whittenbury, R. (1970) Fine structure of metnane and other hydrocarbonutilizing bacteria. *J. Gen. Microbiol.*, 61: 227−230.

Feophilova, E.P. (1974) *Pigments of microorganisms*. − Moscow: Nauka, 218 p.

Gal'chenko, V.F. (1977) *Microbial growth on C_1-compounds*. − Pushchino ONTI NC BI USSR Academy of Sciences, pp. 10−14.

Gal'chenko, V.F., Andreev, A.V. & Trocenko, Y.A. (1986) *Taxonomic identification of methylotrophic bacteria*. − Pushchino ONTI NCBI USSR Academy of Sciences, P. 95.

Ignatov, I. & Mosin, O.V. (2013a) Possible processes for origin of life and living matter with modeling of physiological processes of bacterium *Bacillus subtilis* in heavy water as model system. *Journal of Natural Sciences Research*, 83(8): 132–139.

Ignatov, I. & Mosin, O.V. (2013b) Modeling of possible processes for origin of life and living matter in hot mineral and seawater with deuterium. *Journal of Environment and Earth Science*, 3(14): 103–118.

Karnaukhova, E.N., Mosin, O.V. & Reshetova, O.S. (1993) Biosynthetic production of stable isotope labeled amino acids using methylotroph *Methylobacillus flagellatum. Amino Acids*, 5(1): 125.

Kelly, D.P. (1970) Autotrophy: concepts of lithotrophic bacteria and their organic metabolism. *Ann. Rev. Microbiol.*, 25: 177−185.

Mosin, O.V., Skladnev, D.I. & Shvets, V.I. (1998) Biosynthesis of ^{2}H-labeled phenylalanine by a new methylotrophic mutant *Brevibacterium methylicum. Bioscience, biotechnology, and biochemistry*, 62(2): 225−229.

Mosin, O.V., Ignatov, I., Skladnev, D.A. & Shvets, V.I. (2012) Microbial synthesis of deuterium-labeled *L*-phenylalanine by the facultive methylotrophic bacterium *Brevibacterium meyhylicum* on media with various consentrations of heavy water. *Russian Journal of Biopharmaceuticals*, 4(1): 11–22.

Mosin, O.V., Shvets, V.I., Skladnev, D.I. & Ignatov, I. (2013) Microbial synthesis of ^{2}H-labelled L-phenylalanine with different levels of isotopic enrichment by a facultive methylotrophic bacterium *Brevibacterium methylicum* with RuMP assimilation of carbon. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry*, 7(3): 249−260.

Mosin, O.V., Ignatov, I., Skladnev, D.A. & Shvets, V.I. (2014) A strain of Gram-positive facultative methylotrophic bacteria *Brevibavcterium methylicum* – producer of [^{2}H]phenylalanine. *Drugs development and registration*, 1(6): 58−67.

Mosin, O.V. & Ignatov, I. (2013) Microbiological synthesis of ^{2}H-labeled phenylalanine, alanine, valine, and leucine/isoleucine with different degrees of deuterium enrichment by the Gram-positive facultative methylotrophic bacterium *Brevibacterium methylicum. International Journal of Biomedicine*, 3(2): 132–138.

Mosin, O.V. & Ignatov, I. (2014a) Biological influence of deuterium on procaryotic and eucaryotic cells. *Drugs development and registration*, 2(7): 122−131.

Mosin, O.V. & Ignatov, I. (2014b) Preparation of highly deuterated phenylalanine, alanine, valine and leucine/isoleucine using facultative methylotrophic bacterium *Brevibacterium methylicum. Journal of Medicine, Physiology and Biophysics*, 1: 34−51.

Romanovskaya, V.A. & el Said, M. (1988) Modern state of classification of methylotrophic bacteria. *Microbiology*, 48(2): 97−108.

Suzuhi, T. & Yamahi, T. (1986) Mass production of poli-beta-hydroxybutyric acid by fully automatic fed-batch culture of methylotrophs. *Appl. Microbiol. and Biotechnol.*, 23: 322−329.

Trocenko, Y.A. (1987) *Biochemistry and physiology of methylotrophic microorganisms* / in Collection of scientific articles of USSR Academy of Sciences. – Pushchino: Institute of Biochemistry and Physiology of Microorganisms, P. 25.

Trotsenko, Y.A., Khmelenina, V.N. & Beschastny, A.P. (1995) *The ribulose monophosphate (Quayle) cycle: news and views. Microbial Growth on C1 Compounds* / in: Proceedings of the 8th International Symposium on Microbial Growth on C1 Compounds (Lindstrom M.E., Tabita F.R., eds.). 27 August − 1 September, San Diego, Kluwer Academic Publishers, Boston, pp. 84−86.

Trotsenko, Y.A. & Murrel, J.C. (2008) *Pathways of primary C_1 assimilation and intermediary metabolism. Metabolosm of aerobic methanotrophs* / in: Advances in Applied Microbiology. − London: Academic Press, Elsevier, 63: 205−206.

Tzygankov, Y.D. (1986) *Physiological characterization of methylotrophic bacteria* / in Biology of thermophilic microorganisms. − Moscow: Nauka, pp. 31−50.

Quayle, I.R. (1972) The metabolism of one-carbon compounds by microorganisms. *Adv. Microb. Physiol.*, 7: 119−124.

Whittenbury, R., Phillips, K.C. & Wilkinson, L.F. (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J. Gen Microbiol.*, 61: 205−210.

Whittenbury, R., Davies, S.L. & Davey, S.I. (1970) Exospores and cysts formed by methane utilizing bacteria. *J. Gen. Microbiol.*, 61: 219−227.