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Role of monooxygenase reaction during assimilation of non-growth substrates by methanotrophs

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Abstract

Methanotrophic bacteria utilize only methane (some species methanol) as a source of carbon and energy. They can co-metabolize a number of non-growth substrates due to the low specificity of methane monooxygenase (MMO). The reducer for monooxygenation is obtained during dehydrogenation of intermediates of methane oxidation. During growth on methane, co-metabolism of non-growth substrates leads to exhaustion of the methanotroph energy sources, accumulation of toxic products and growth cessation. To evaluate a role of the monooxygenation process in the metabolism of methanotrophs, co-metabolism of methanol and a substrate of MMO (ethane or carbon monoxide) was studied during the growth of *Methylomonas rubra* 15sh and *Methylococcus thermophilus* 111p. These organisms, being grown at low copper content, did not oxidize naphthalene. Thus, they possess particulate MMO, which can use reducing equivalents at the level of methanol dehydrogenase. Methanol did not support growth of *Mc. thermophilus* 111p. Growth of *Mm. rubra* 15sh on methanol was inefficient and was accompanied by accumulation of formaldehyde. When a second substrate (ethane or carbon monoxide) was added into the gas phase (5–20 vol.%), the growth of *Mc. thermophilus* 111p occurred, and the yield from methanol of *Mm. rubra* 15sh was enhanced. At low methanol concentration (to 12.5 mM), the yield of the *Mm. rubra* 15sh biomass from methanol was proportional to the amount of the second substrate co-oxidized, and the whole methanol utilized was converted into biomass. Theoretical calculations showed that such high efficiency of growth could be achieved when MMO received reducing equivalents from methanol dehydrogenase and, moreover, electron transport chain from NADH to MMO contained a proton translocating segment. Thus, monooxygenation of the MMO substrate was profitable for the methanotroph metabolism. During the growth of methanotrophs studied on methanol plus ethane or carbon monoxide, the major part of electrons of methanol utilized was used for the monooxygenation of the MMO substrate. For methanotrophs, this allows to consider the monooxygenation process as the energy yielding mechanism of the electron transport to oxygen.

Keywords: Methanotrophs; *Methylomonas rubra*; *Methylococcus thermophilus*; Co-metabolism; Methanol; Carbon monoxide; Ethane; Methane monooxygenase

1. Introduction

Methanotrophs represent a group of methylotrophic bacteria assimilating only methane

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(in a number of cases, methanol). Metabolism of methane, as well as other one-carbon compounds, includes a number of unique biochemical processes, which determine biotechnological potential of methylotrophs [1,2]. Hydrocarbons other than methane and their halogen substituted analogues are not assimilated by methanotrophs as sole sources of carbon and energy, however, they can be oxidized by the cells in conditions of co-metabolism. Co-metabolism requires methane, or non-growth energy yielding substrate, and is due to broad substrate specificity of methane monooxygenase (MMO) [3–11]. Reducing equivalents for MMO can be obtained from dehydrogenation of the oxidation intermediates of methane (methanol, formaldehyde, formate), ethane (ethanol, acetaldehyde), propane (propanol) [12]. Acetate, propanal and other products of co-metabolism of the methane substrate analogues are accumulated in the medium [13].

Methanotrophs oxidize methane to methanol via MMO [1]. Two distinct forms of this enzyme exist in the same organisms, a soluble form (sMMO) and membrane-bound, particulate form (pMMO) [14]. Growth at high copper to biomass ratios produces a pMMO, whereas at low copper to biomass ratios the sMMO predominates. The sMMO uses as a reductant only NADH, whereas pMMO can use electrons driven from alcohols. The latter case is more efficient for biomass biosynthesis [15,16]. Co-metabolism usually is terminated due to accumulation of toxic products, or due to the lack of biosynthetic processes. For maintenance of co-metabolism for a long time, constant sources of reductant for monooxygenation and carbon for the cell growth are required. Methanol is a good substrate for this purpose. Many methanotrophs grow on methanol, though efficiency of biosynthesis in this case is low.

Aims of this study were to evaluate the reasons of low efficiency of the methanol assimilation and to model variants of co-metabolism, providing for the cell growth, joint consumption of methanol and non-assimilating substrate oxidizing by MMO (ethane or CO). Two species of methanotrophic bacteria *Methylomonas rubra* 15sh (grows on methanol) and *Methylococcus thermophilus* 111p (unable to grow on methanol) were investigated. Peculiarities of assimilation of non-growth substrates (methanol with ethane or with carbon monoxide) by methanotrophs demonstrated a role of MMO as a main reaction of transfer of the substrate electrons to oxygen.

2. Experimental

2.1. Objects of study

Strains of obligate methane oxidizing bacteria (methanotrophs) *Mm. rubra* 15sh and *Mc. thermophilus* 111p as well as *Methylosinus trichosporium* OB3b were used [17,18].

2.2. Methods of cultivation

The following mineral medium was used for the methanotroph batch cultivation, (g/l): $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.4; $K_2HPO_4 \times 3H_2O$, 0.4; NaCl, 0.3; $MgSO_4 \times 7H_2O$, 0.3; $CaCl_2 \times 6H_2O$, 0.02; $FeCl_3 \times 6H_2O$, 0.001; microelements (μg/l): $CuSO_4 \times 5H_2O$, 250 (1250 for pMMO expression); $MnSO_4 \times 4H_2O$, 5; $ZnSO_4 \times 7H_2O$, 35; H_3BO_3, 5; $Na_2MoO_4 \times 2H_2O$, 5; $CoSO_4 \times 7H_2O$, 15; pH 6.7. For agar medium, 15 g/l of Bacto-Agar Difco was added. Inoculated plates were incubated at optimal temperature (*Mm. rubra* 15sh, 30°C; *Mc. thermophilus* 111p, 50°C) about 7 days in methane–air (1:1) atmosphere. Cultivation in flasks in liquid medium under methane–(CO– or ethane–) air atmosphere was performed on a rotary shaker (200 rpm) during 48–72 h. Cell concentration was determined by measuring of optical density at 600 nm and expressed as dry weight. Microbiological purity of cultures was checked by spreading over the LB agar and by microscopy.

2.3. Determination of substrates and products of metabolism.

Concentration of components of a gas phase (O_2,CH_4, C_2H_6, CO, CO_2), methanol, ethanol and acetaldehyde in culture liquid were determined by gas chromatography [19]. Formaldehyde was determined by reaction with acetylacetone reagent [20]. Acetate was determined with acetate kinase [21]. Methane oxidation rate in cell suspensions was determined polarographically [19]. Rate of naphthalene oxidation by cells was followed by formation of α-naphthol, which was determined with *o*-dianisidine [22].

All the assays were carried out in triplicate.

3. Results

3.1. Determination of the MMO form in methanotrophs

The sMMO and pMMO synthesis in a number of species of methanotrophs is regulated by the copper concentration in the cultivation medium [14]. The determination of the sMMO activity in the whole cells is based on ability of this enzyme to oxidize naphthalene (in contrast to pMMO). Cells of *Mm. rubra* 15sh and *Mc. thermophilus* 111p grown in the copper deficient medium did not oxidize naphthalene (Table 1). Apparently, in these organisms pMMO is constitutive. In accordance with published data [22], the *Ms. trichosporium* OB3b cells similarly grown in the copper deficient medium were able to oxidize naphthalene.

3.2. Growth on methanol

Growth of *Mm. rubra* 15sh on methanol as a sole source of carbon and energy was characterized by low efficiency (Y_C) and was accompanied by accumulation of formaldehyde in the medium (Table 2). With the increase of methanol concentration Y_C reduced, and concentration of formaldehyde raised. *Mc. thermophilus* 111p did not grow on methanol, though various cultivation conditions were tested: concentration of methanol in a liquid medium from 2.5 to 50 mM; incubation on agar medium in methanol vapor; sources of nitrogen $(NH_4)_2SO_4$ and KNO_3; O_2 concentration in gas phase from 5% to 20%; cultivation at the presence of carbon dioxide (5% CO_2 in the gas phase and 0.5 g/l $NaHCO_3$ in medium); temperature from 42°C to 55°C. After 3–4 days of cultivation, formaldehyde was detected (up to 1 mM) in methanol containing medium inoculated with *Mc. thermophilus* 111p. Thus, methanol was oxidized by cells introduced; however, biomass did not increase. The reason for formaldehyde accumula-

Table 1
Rates of the methane and naphthalene oxidation by cell suspensions of methanotrophs grown in media with different copper content
Cell densities in all experiments were 0.2–0.4 mg ml^{-1} of reaction mixture. Data are the average results of tree experiments.

Methanotroph species	Cu^{2+} in growth medium (μM)	Rate of methane oxidation[a] (nmol min^{-1} mg^{-1})	Rate of α-naphthol formation[b] (nmol min^{-1} mg^{-1})
Mm. rubra 15sh	1	108.1	0
	5	76.7	0
Mc. thermophilus 111p	1	83.5	0
	5	120.6	0
Ms. trichosporium OB3b	1	46.6	6.3
	5	104.7	0

[a]Activity was measured in 0.03 M tris–HCl buffer pH 7.0 with 0.17 mM of methane polarographically.
[b]Activity was measured in 0.03 M tris–HCl buffer pH 7.0 with crushed naphthalene (50 mg per 2.5 ml of reaction mixture) and 10 mM of sodium formate.

Table 2
Stoichiometry of biomass synthesis and formation of the oxidation products during methanotroph growth on methanol and methanol + CO
Data are the average results of three experiments.

Initial substrate concentration		Substrate utilized (mmol per 1 l of culture liquid)		Biomass (g/l)	Y_{CH_3OH} (g of biomass per g of methanol)	Y_C[a] (g C of biomass per g C of methanol)	Accumulated products (mmol per 1 l of culture liquid)	
CO (vol.%)	Methanol (mM)	CO	CH_3OH				HCOH	CO_2
Mm. rubra 15sh								
0	12.5	–[b]	6.25	0.07	0.35	0.44	0.83	2.68
0	25	–	18.75	0.20	0.33	0.42	1.67	9.19
0	75	–	21.88	0.11	0.16	0.20	2.5	15.1
10	12.5	13.65	10.90	0.227	0.65	0.82	0	15.6
10	25	18.75	18.75	0.36	0.60	0.75	0	23.4
10	75	7.69	18.75	0.18	0.30	0.38	1.17	18.2
Mc. thermophilus 111p								
0	12.5	–	3.2	0	0	0	0.7	2.50
0	25	–	9.26	0	0	0	0.83	8.43
10	12.5	13.3	8.75	0.16	0.57	0.72	0	15.6
10	25	10.5	13.13	0.19	0.45	0.57	0.4	15.8

[a] $Y_C = (32/25.5)\ Y_{CH_3OH}$, where 32 is the methanol molecular weight, 25.5 is the molecular weight of compound $CH_2O_{0.5}\ N_{0.25}$, reflecting the methanotroph biomass composition.
[b] CO was not added.

tion could be the ability of MMO to oxidize methanol [23,24]. Apparently, formaldehyde-yielding activity (two enzymes produce this intermediate, MMO and methanol dehydrogenase (MDH)) was higher than the activity of formaldehyde oxidation to CO_2 and its utilization for biosynthesis of cell constituents. Formaldehyde oxidation in methanotrophs is limited by the activity of NADH oxidase [25]. Utilization of surplus reducing equivalents (NADH), appearing during growth on methanol, would be possible if alternative to NADH oxidase reducer scavenger will function. Monooxygenation of any substrate (another than methanol) by MMO can be such reaction utilizing formaldehyde electrons.

3.3. Growth on methanol + ethane

Mc. thermophilus 111p (not assimilating methanol as a sole source of carbon and energy) was able to grow on methanol (20 mM) in the presence of ethane (20 vol.%). Formaldehyde did not accumulate (data not shown).

Growth experiments with *Mm. rubra* 15sh (able to grow on methanol) have shown that the efficiency of methanol conversion into biomass (Y_C) enhanced in the presence of ethane (Fig. 1, see Table 2 for growth on methanol). The ratio of products of ethane co-oxidation depended on the methanol concentration (Fig. 1A), and to a lesser extent on the ethane concentration (ethane oxidation product concentrations were higher at 10 and 20 vol.% of ethane in the gas phase, data not shown). Maximal yield was observed at low concentrations of methanol (up to 10 mM); ethane was oxidized to acetaldehyde and acetate (Fig. 1A), proportionally to consumed methanol in the ratio, close to 1:1 (Fig. 1C). The yield from methanol indicated that carbon of utilized methanol was spent for synthesis of biomass and, probably, some additional carbon was received from ethane (presumably, acetate can be assimilated [26–30]). As carbon dioxide was not produced, practically the whole methanol was converted to biomass. Thus, a sole reaction of methanol conversion coupled with the ethane monooxygenation could be oxidation of methanol by MDH. Oxidation of the ethane

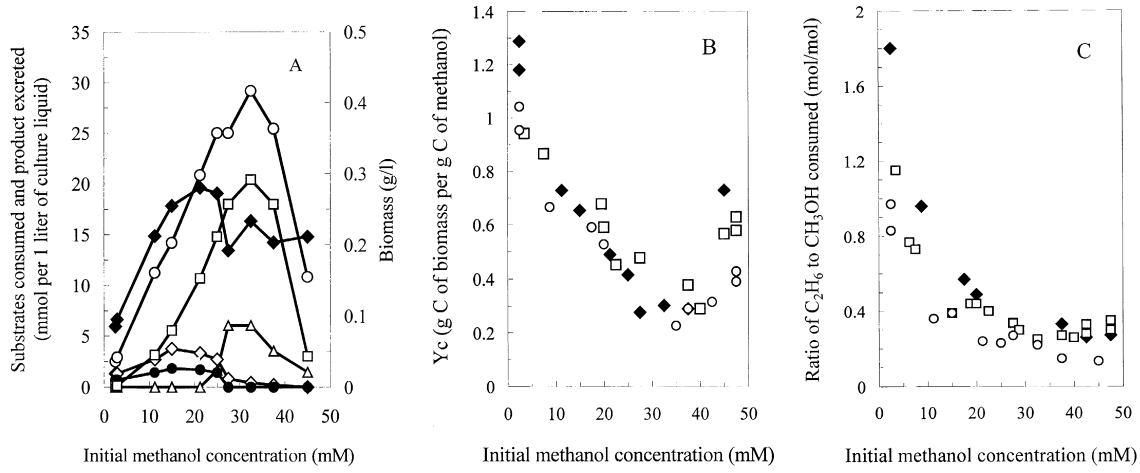

Fig. 1. Effect of the initial methanol concentration on the growth efficiency of *Mm. rubra* 15sh cultures utilizing methanol + ethane. (A) Biomass (♦),CO_2 (□), ethanol (△), acetaldehyde (◇), and acetate (●) produced, and methanol (○) consumed at 5 vol.% of ethane. (B) Yield (Y_C) from methanol and (C) the ratio of ethane to methanol consumed for the initial ethane concentration in the gas phase: 5 (♦); 10 (□); and 20 (○) vol.%. The Y_C values were calculated as described in the footnote of Table 2. Each point represents the average data obtained for three independent experiments.

monooxygenation product ethanol via acetaldehyde to acetate also yields a reducing power for MMO. At higher concentrations of methanol (above 30 mM), oxidation of ethanol competed with methanol for MDH. As a result, ethanol was excreted (Fig. 1A). In these cases, an intensive oxidation of methanol to carbon dioxide was observed, and efficiency of biomass synthesis was low. Ethane co-oxidation remained proportional (1:1) to the quantity of methanol converted into biomass. As ethanol was not oxidized further, an energy yielding process was oxidation of methanol to carbon dioxide. At methanol concentrations above 40 mM, it competed with ethane for MMO, which resulted in the decrease of ethanol accumulation and some increase of biomass yield (Fig. 1A,B).

3.4. Growth on methanol + CO

When ethane was used as a substrate for monooxygenation, it was impossible unequivocally to evaluate the significance of the methanol electron acceptance in the monooxygenase reaction for the growth process. During co-metabolism, assimilation of some ethane carbon or/and energy was possible. For demonstration of a role of the monooxygenation reaction in energy metabolism, carbon monoxide was used as another methane substrate analogue [31]. Carbon monoxide possesses high affinity to MMO [13], and the product of its monooxygenation (carbon dioxide) is not toxic and does not serve more as a source of energy. The CO monooxygenation is coupled to the consumption of the reducing equivalents, therefore if transport of electrons to MMO is not coupled with generation of transmembrane electrochemical gradient, the given process can only decrease the efficiency of methanol assimilation. However, the growth of methanotrophs at low concentrations of methanol (12.5–25 mM) in the presence of CO was characterized by a higher yield of biomass than when methanol was a single substrate (Table 2). Growth of *Mc. thermophilus* 111p under conditions of the methanol + CO co-metabolism was also possible (Table 2).

Stoichiometry of growth process depended on concentration of methanol (Table 2). During growth of *Mm. rubra* 15sh, the ratio of methanol and CO consumed decreased from 1.125 (at 12.5 mM of methanol) to 0.41 (at 75 mM of

methanol). This correlated with the decrease of Y_C. Data from Table 2 show that the more oxidized CO during growth, the higher the yield from methanol was. Formaldehyde was accumulated only at concentrations of methanol above 60 mM, due to competition of methanol and CO for MMO. Thus yield from methanol decreased. Similar regularities were observed during the growth *Mc. thermophilus* 111p on methanol + CO (Table 2).

3.5. Elucidation of the MMO role

The assimilation of a substrate via monooxygenation should be less effective due to the expense of the reducer. During co-metabolism, the biomass yield was unusually high (at low concentrations of methanol). This testifies to the important role of monooxygenase reaction in the metabolism of methanotrophs. On the basis of the growth stoichiometry (see Table 2), we built equations demonstrating possible fate of the consumed substrates in order to elucidate mechanisms benefiting from the monooxygenase reaction.

Stoichiometry of the *Mm. rubra* 15sh growth on methanol + CO at the methanol concentration 12.5 mM can be described by the following equation (formula of biomass was built, taking into account that the methanotroph cells contain 13% of nitrogen; H^+, ADP, PO_4^{3-}, NAD^+, NH_3, and H_2O formed were omitted):

$$CH_3OH + 1.25\,CO + 1.25\,O_2 \rightarrow 0.82\ \underset{\text{Biomass}}{CH_2O_{0.5}\,N_{0.25}} + 1.43\,CO_2$$

The process described by the given equation consists of the following stages of transformation of substrates:

1. CO monooxygenation: $1.25\ CO + 1.25\ O_2 + 2.5\ [H] \rightarrow 1.25\ CO_2$;
2. Dehydrogenation of methanol: $CH_3OH \rightarrow HCOH + 2\ [H]$
3. Part of formaldehyde was oxidized to CO_2 to yield additional reducing equivalents [H] necessary for reactions (1) minus (2):

$$0.125\,HCOH + 0.125\,H_2O \rightarrow 0.125\,CO_2 + 0.5\,[H]$$

4. Synthesis of biomass from the rest of formaldehyde via ribulose monophosphate pathway (amounts of NADH and ATP required were taken from [32]):

$$0.82\,HCOH + 0.11\,NADH + 2.5\,ATP \rightarrow 0.82\,\underset{\text{Biomass}}{CH_2O_{0.5}N_{0.25}}$$

$$0.055\,HCOH + 0.055\,H_2O \rightarrow 0.055\,CO_2 + 0.11\,NADH$$

$$0.875\,HCOH + 2.5\,ATP \rightarrow 0.82\,\underset{\text{Biomass}}{CH_2O_{0.5}\,N_{0.25}} + 0.055\,CO_2$$

From Eq. (4) one can see that a very small part (5.5%) of metabolized methanol provides for NADH (at the level of formaldehyde) necessary for biosynthesis. Practically all electrons of methanol, released during the dehydrogenase reactions, as reducing equivalents [H] were accepted at CO monooxygenation (Fig. 2), and their transport to MMO should ensure the synthesis of ATP, necessary for biosynthetic reactions. There was no NADH available for oxida-

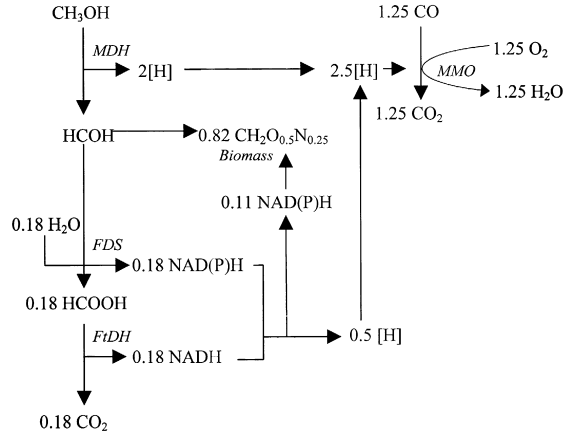

Fig 2. Possible stoichiometry of methanol + CO assimilation during growth of *Mm. rubra* 15sh on methanol + CO at the initial methanol concentration 12.5 mM. MDH, methanol dehydrogenase; MMO, methane monooxygenase; FDS, formaldehyde dehydrogenating system; FtDH, formate dehydrogenase; [H], reducing equivalents.

tion via usual electron transport chain. During assimilation of 1 mol of methanol, 2.5 electrons were transferred and synthesis of 2.5 mol of ATP was necessary. It is considered that proton ATPase synthesizes 1 mol ATP per 2 mol of protons translocated. Taking this into account, necessary efficiency of ATP synthesis can be achieved only when MDH will create an electrochemical gradient as a redox arm, giving the methanol electrons to MMO. In addition, transport of electrons from NADH to MMO should possess a proton translocating segment, in this case MMO should use protons from cytoplasm.

4. Discussion

Cells of *Mm. rubra* 15sh and *Mc. thermophilus* 111p grown under copper deficiency did not oxidize naphthalene. This indicated that these organisms possess constitutive pMMO that is much more efficient than sMMO.

Methanotroph *Mc. thermophilus* 111p was not able to grow on methanol. During growth of methanotrophs on methanol as a sole source of carbon and energy, part of it is oxidized by MMO, and in some species (*Ms. trichosporium* OB3b) the methanol monooxygenation is the basic sink of methanol electrons to oxygen [24]. If methanol monooxygenation does not proceed (for example, when the MMO inhibitor, acetylene, is present), the growth is possible only of those species which possess an alternative system of transport of electrons to oxygen (*Mm. rubra* and *Mc. capsulatus*) [24].

A possibility of growth of *Mc. thermophilus* 111p on two non-growth substrates (methanol + CO, or methanol + ethane) was shown. For *Mm. rubra* 15sh, a methanotroph capable to grow on methanol, the high efficiency of the methanol carbon and energy conversion can be achieved only when the major part of its electrons will be accepted during monooxygenation of a MMO substrate.

The joint assimilation of methanol and the substrate analogue of methane (ethane or CO) by methanotrophs was due to coupling of the reactions of methanol dehydrogenation with the second substrate monooxygenation, which provides for more efficient utilization of the methanol energy and carbon and indicates the contribution of monooxygenase reaction into the energy metabolism. The more monooxygenated ethane or CO during the growth, the higher the yield of biomass was; thus the efficiency of energy metabolism.

Mm. rubra 15sh yield from methanol and the ratio of ethane to methanol consumed depended on the concentration of methanol but not ethane (see Fig. 1). The competition of methanol and ethane (or CO) for MMO determined the efficiency of conversion of methanol by cells. For high yield, methanol should not be oxidized by MMO, because duplication of the MDH function resulted in strengthening of the formaldehyde flow. This formaldehyde was not used by methanotrophs and was excreted. Excretion of formaldehyde when MMO activity was low (without a substrate with high affinity to MMO) suggested a lack of energy (ATP) for involvement of formaldehyde into biomass synthesis. Introduction of a substrate of MMO intensified the monooxygenase reaction and enhanced the efficiency of methanol conversion.

The joint assimilation by methanotrophic bacteria of methanol and substrate analogue of methane (ethane or CO) represents a variant of co-metabolism, which results in more intensive and efficient growth of cells. This has allowed to determine this phenomenon as ‘‘syntabolism’’. Application of a syntabolism principle (additional introduction of methanol) can avoid an inhibition of the methanotroph growth on natural gas with high contents of ethane, caused by co-oxidation of methane and ethane.

Thus, the basic peculiarity of energy metabolism of methanotrophic bacteria is the participation of MMO in the acceptance of a major part of electrons of assimilated substrate, and in the case of pMMO this process should be connected to the generation of energy in the form of transmembrane electrochemical gradi-

ent. The second mechanism of creation of electrochemical gradient can be realized during methanol dehydrogenation by periplasmic MDH. Therefore, mechanisms of energy generation in methanotrophs differ much from those in heterotrophic microorganisms. The absence of the ability of all methanotrophs tested to use multicarbon substrates as sole sources of carbon and energy is mainly caused by inability of the systems of electron transport from NADH to oxygen to provide cells with energy. The latter is apparently the general and the most essential reason of obligate dependence from sources of carbon and energy of microorganisms with specific mechanisms of their transformation, photo-, litho- and methylotrophs.

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