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Characterization of Recombinant PPi-Dependent 6-Phosphofructokinases from *Methylosinus trichosporium* OB3b and *Methylobacterium nodulans* ORS 2060

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Abstract—The properties of the purified recombinant PPi-dependent 6-phosphofructokinases (PPi-PFKs) from the methanotroph *Methylosinus trichosporium* OB3b and rhizospheric phytosymbiont *Methylobacterium nodulans* ORS 2060 were determined. The dependence of activities of PPi-PFK-His_6-tag from *Ms. trichosporium* OB3b (6 × 45 kDa) and PPi-PFK from *Mb. nodulans* ORS 2060 (4 × 43 kDa) on the concentrations of substrates of forward and reverse reactions conformed to Michaelis–Menten kinetics. Besides fructose-6-phosphate, the enzymes also phosphorylated sedoheptulose-7-phosphate. ADP or AMP (1 mM each) inhibited activity of the *Ms. trichosporium* PPi-PFK but did not affect the activity of the *Mb. nodulans* enzyme. Preference of PPi-PFKs to fructose-1,6-bisphosphate implied a predominant function of the enzymes in hexose phosphate synthesis in these bacteria. PPi-PFKs from the methylotrophs have low similarity of translated amino acid sequences (17% identity) and belong to different phylogenetic subgroups of type II 6-phosphofructokinases. The relationship of PPi-PFKs with microaerophilic character of *Ms. trichosporium* OB3b and adaptation of *Mb. nodulans* ORS 2060 to anaerobic phase of phytosymbiosis are discussed.

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In most microorganisms a key stage of glycolysis – phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (FBP) – is catalyzed by allosterically regulated ATP-dependent 6-phosphofructokinase (ATP-PFK, EC 2.7.1.11). However, a number of pro- and eukaryotes, including phototrophic bacteria (*Rhodospirillum rubrum*), anaerobes, parasitic microorganisms (*Treponema pallidum*, *Entamoeba histolytica*, *Trichomonas vaginalis*), *Euglena gracilis* and higher plants, have pyrophosphate-dependent 6-phosphofructokinase (PPi-PFK, EC 2.7.1.90), which is homologous to ATP-PFK [1]. The reaction catalyzed by PPi-PFK is reversible, thus suggesting participation of the enzyme in both glycolysis and gluconeogenesis.

The origin and metabolic consequences of replacement of ATP by PPi in the central reaction of glycolysis are not obvious. Alternative hypotheses have been proposed in which the PPi-PFK is considered as an attribute of an "ancient metabolism" that has been conserved as a rudiment in a small number of species, or, conversely, as result of mutations of ATP-dependent enzymes during adaptation to anaerobic conditions [1-3].

PPi-PFK was found in obligate methanotrophs – a specialized group of aerobic bacteria using methane as carbon and energy source and not able to grow on multicarbon compounds, as well as in a small number of facultative methylotrophic bacteria growing on methanol and some carbohydrate substrates [4-6].

Three basic cyclic pathways of primary assimilation of C_1 compounds and synthesis of cell components have been shown to function in methanotrophic and methy-

Abbreviations: ATP-PFK, ATP-dependent 6-phosphofructokinase; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani medium; PFK, 6-phosphofructokinase; PPi-PFK, pyrophosphate-dependent 6-phosphofructokinase; PRK, phosphoribulokinase; RuBisCO, ribulose-1,5-bisphosphatecarboxylase/oxygenase; RuMP, ribulose monophosphate; SBPase, sedoheptulose-1,7-bisphosphatase; S7P, sedoheptulose-7-phosphate.

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lotrophic bacteria. In the ribulose monophosphate (RuMP) cycle, hexulose-6-phosphate as the first product is formed by condensation of formaldehyde and ribulose-5-phosphate. At this stage in methanotrophs with the RuMP-cycle two pathways of C_6 phosphosugar cleavage for synthesis of C_3 compounds are realized: Entner–Doudoroff and Embden–Meyerhof–Parnas (glycolysis). In methylotrophs with the serine pathway, C_3 compound (serine) is the primary biosynthetic product formed by condensation of formaldehyde and glycine. Some methylotrophic bacteria assimilate reduced C_1 compounds, after oxidation to CO_2, via the Calvin cycle in which 3-phosphoglycerate is produced as a first stable intermediate. Thermotolerant methanotrophs of the *Methylococcus* and *Methylocaldum* genera assimilate C_1 compounds via the three simultaneously operating pathways listed above [7-9]. Thus, in methylotrophic bacteria the functional significance of glycolysis/gluconeogenesis differs depending on the pathway of C_1 assimilation. Therefore, these bacteria are convenient model organisms for elucidating the role of PPi-PFK in carbon metabolism.

Earlier, we characterized PPi-PFKs from methanotrophs with the RuMP-cycle (*Methylomonas methanica* 12 and *Methylomicrobium alcaliphilum* 20Z), and also from *Methylococcus capsulatus* Bath [10-13]. Based on differences in the enzyme properties and the results of analysis for the genomic sequences of these bacteria, several important functions of PPi-PFK in carbon and energy metabolism were proposed. However, this enzyme from the serine pathway methylotrophic bacteria has not been investigated.

The aim of this study was to purify and characterize the recombinant PPi-PFKs from the obligate methanotroph *Methylosinus trichosporium* OB3b and the facultative phytosymbiont *Methylobacterium nodulans* ORS 2060 assimilating C_1 compounds via the serine pathway.

MATERIALS AND METHODS

Bacteria and growth conditions. *Methylosinus trichosporium* OB3b^T was grown under a methane–air atmosphere (1 : 1) in mineral medium at 28°C with shaking (200 rpm) [14]. *Methylobacterium nodulans* ORS 2060^T was cultivated at 28°C in a flask with mineral medium containing (g/liter): KH_2PO_4 (2.0), $(NH_4)_2SO_4$ (2.0), NaCl (0.5), $MgSO_4 \cdot 7H_2O$ (0.025), $FeSO_4 \cdot 7H_2O$ (0.002), pH 7.2, in the presence of methanol (0.5%) or L-arabinose (1 g/liter) with shaking (200 rpm) [15]. *Escherichia coli* BL21(DE3) was grown in liquid Luria–Bertani (LB) medium at 150 rpm and 37°C. Ampicillin and chloramphenicol (100 μg/ml) were added for growth of *E. coli* cells containing plasmids.

Cloning of *pfp* genes and PP_i-PFK purification. Chromosomal DNAs from *Mb. nodulans* and *Ms. trichosporium* were isolated as described previously [16]. The *pfp* gene from *Mb. nodulans* ORS 2060 (Mnod_0681) was amplified by PCR using primers PFKnod-N and PFKnod-C (Table 1) designed on basis of the sequence available in GenBank (accession number NC_011894). The PCR-product was cloned into the expression vector pHUE, which yielded a heterologous protein fused with ubiquitin [17]. Cells of *E. coli* BL21(DE3) carrying an additional plasmid pT-groE were transformed by the resulting plasmid pHUE:*pfpnod*. The transformed *E. coli* cells were grown overnight at 37°C in LB medium, transferred into fresh LB medium containing 100 μg/ml ampicillin and chloramphenicol, and cultivated to A_{600} = 0.6-0.7. The expression of the protein was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to the final concentration of 1 mM. After 5 h incubation at 27°C, the cells were harvested by centrifugation (6000*g*, 20 min at 4°C) and stored at –20°C. The His_6-Ub-PPi-PFK was purified by affinity chromatography on a Ni^{2+}-NTA column as described earlier [11]. After treatment with the deubiquitinating enzyme Usp2-His_6 for 1 h at 4°C, PPi-PFK was kept in 40% glycerol at –20°C. Before characterization, an aliquot of the protein was dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl, and purified again on a Ni^{2+}-NTA column.

The *pfp* gene (EFH03142) from *Ms. trichosporium* OB3b was amplified from the chromosomal DNA using primers pfkOB3b-N and pfkOB3b-C (Table 1) that were designed based on the sequence available in GenBank (accession number ADVE01000022). The PCR-product was cloned in the expression vector pET22b$^+$. Cells of *E. coli* BL21(DE3) carrying the plasmid pT-groE were transformed by the resulting plasmid pET22b:*pfpOB3b*. The transformed *E. coli* cells were grown overnight in LB medium at 37°C, transferred into fresh LB medium containing 100 μg/ml ampicillin and chloramphenicol, and grown to A_{600} = 0.6-0.7. For protein expression, 1 mM IPTG was added into the culture, which was then incubated at 18°C for 10 h. The cells were harvested by cen-

Table 1. Primers used

Primer	Nucleotide sequences (5′-3′)
PFKnod-N	TAGGATCCATGACCACAGCCAAAGT (*Bam*HI)
PFKnod-C	TCAAGCTTAGGCGTGCGGCTGCCCGAT (*Hind*III)
pfkOB3b-N	TTCATATGTCCGACATCGACTTCTCCAA (*Nde*I)
pfkOB3b-C	TTAAGCTTCTTTTTCAAAATCTTGGCGACAT (*Hind*III)

Note: Sites of restriction endonucleases (indicated in parentheses) are underlined.

Table 2. Range of substrate concentrations used for determination of kinetic characteristics of PPi-PFK

Substrate	*C*, mM	
	Mb. nodulans	*Ms. trichosporium*
F6P	0.10-3.00	0.06-2.00
S7P	0.07-3.75	0.03-2.50
FBP	0.02-3.00	0.03-1.00
PP_i	0.01-1.00	0.01-1.00
P_i	0.40-30.0	0.90-45.0
Mg^{2+}*	0.03-5.00	0.02-2.50
Mg^{2+}**	0.15-5.00	0.15-5.00

* Forward reaction.
** Reverse reaction.

trifugation (6000*g*, 20 min) and stored at −20°C. PP_i-PFK-His_6-tag was purified by affinity chromatography on a Ni^{2+}-NTA column. The enzyme was stored in 3.2 M $(NH_4)_2SO_4$ at 4°C. Before use, an aliquot of the protein was dialyzed against 20 mM Tris-HCl buffer, pH 7.5.

The purified enzymes were analyzed by electrophoresis in 12% SDS-polyacrylamide gel [18]. β-Galactosidase (116.0 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), ribonuclease Bsp98I (25.0 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa) were used as protein molecular mass markers.

Determination of the PPi-PFK molecular mass. To determine the molecular mass of the native enzymes, a nondenaturing gel electrophoresis was performed using the pore-limiting gradient of polyacrylamide (4-30%) [19] and a set of protein markers (Pharmacia, USA) containing ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and BSA (67 kDa). Additionally, the *Ms. trichosporium* OB3b enzyme was gel filtered on a Ultrogel AcA 34 column equilibrated with 20 mM Tris-HCl buffer, pH 7.2, containing 100 mM NaCl. The reference proteins were ferritin, amylase (200 kDa), alcohol dehydrogenase (150 kDa), and BSA (Sigma, USA).

PPi-PFK activity assay. The activities of PPi-PFK in the forward and reverse reactions were determined by registering FBP or F6P formation with coupling enzymes using earlier described methods [12]. Cell-free extracts of *Mb. nodulans* were prepared as described [14].

To study the pH dependence of PPi-PFK activity, 50 mM imidazole-HCl (pH 6.0-8.0) and Tris-HCl (pH 7.0-8.9) buffers were used. Potential effectors (pyruvate, citrate, serine, phosphoglycerate, phosphoenolpyruvate, ATP, ADP, AMP, $NADP^+$, NAD^+, NADPH, glucose-6-phosphate, glucose-1-phospate, fructose-1-phosphate, ribose-5-phosphate or glyceraldehyde-3-phosphate) were added to the reaction mixture to the final concentration of 1 mM. To test the ability of some divalent metals to replace Mg^{2+}, aqueous stock solutions of $BaCl_2$, $MnCl_2$, $CoCl_2$, or $CuCl_2$ were added to final concentration of 0.01-1 mM. To test the potential acceptors of the phosphoryl group, glucose-6-phosphate, glucose-1-phosphate, fructose-1-phosphate, mannose-6-phosphate, ribulose-5-phosphate, ribose-5-phospate, ribose-1-phosphate, erythrose-4-phosphate, and glyceraldehyde-3-phosphate were used at the final concentration of 5 mM, and the enzyme activity was determination by measuring of the orthophosphate concentration as described earlier [11]. This method was also applied for studies of the dependence of the enzyme activity on temperature. The apparent K_m and A_{max} values were calculated using SigmaPlot (v.10). Kinetic parameters of PPi-PFK were determined at different concentrations of one substrate and saturating concentrations of the other (Table 2).

Protein concentrations were assayed by a modified Lowry method [20] using BSA as a standard.

Sequence analysis. Sequences from the NCBI database (http://www.ncbi.nlm.nih.gov) were obtained using BLAST. Alignments of amino acid sequences of PFKs were generated using the CLUSTALX software (v.1.8) [21]. Minor corrections of the alignments were made manually. Phylogenetic analysis was performed using the MEGA 4 software and the Neighbor-Joining model [22]. The *pfp* gene sequences from *Mm. alcaliphilum* 20Z and *Methylocaldum szegediense* O-12 were obtained from their partially completed genomes (http://www.genoscope.cns.fr/externe/English/corps_anglais.html).

DNA manipulations. Plasmid isolation, restriction, agarose gel electrophoresis, ligation, and transformation of *E. coli* cells were performed according to standard methods [23] using restriction enzymes, T4 DNA-ligase, *Pfu* DNA-polymerase, and dNTP mixture manufactured by Fermentas (Lithuania).

RESULTS AND DISCUSSION

Cloning of *pfp* genes and PPi-PFK purification. The *pfp* genes encoding PPi-PFK were amplified from the genomic DNAs. The PCR-product from *Ms. trichosporium* was cloned into the bacterial vector pET22b(+) designed for expression of C-terminal His_6-tagged fusion proteins under control of the T7 promoter. The *Mb. nodulans* PCR-product was cloned into the expression vector pHUE, which yields authentic proteins without His-tags. Cells of *E. coli* BL21(DE3) carrying the additional plasmid pT-groE with chaperone-encoding genes were transformed by the recombinant plasmids. This yielded the

recombinant proteins in the soluble fraction. PPi-PFK-His_6 from *Ms. trichosporium* and His_6-Ub-PPi-PFK from *Mb. nodulans* were purified from *E. coli* extracts by affinity chromatography on a Ni^{2+}-NTA column in a single step. Then *Mb. nodulans* His_6-Ub-PPi-PFK was treated by the deubiquitinating enzyme Usp2 and again purified on the Ni^{2+}-NTA column. As a result, electrophoretically homogenous preparations of the enzymes were obtained (Fig. 1).

Biochemical characterization of PPi-PFK. The purified proteins had electrophoretic mobility in the SDS-polyacrylamide gel corresponding to the theoretically calculated masses of the subunits (43.2 and 44.4 kDa) (Fig. 1). According to native gradient electrophoresis, the molecular mass of the *Mb. nodulans* enzyme is ~180 kDa. Therefore, the enzyme is a homotetramer. The homotetrameric structure of PPi-PFK was testified for *Amycolatopsis methanolica* and *Mm. alcaliphilum* 20Z enzymes [5, 12]; however, most of the studied microbial PPi-PFKs are homodimers. Gel filtration and native gradient electrophoresis revealed that the *Ms. trichosporium* enzyme is a homohexamer with molecular mass 270 kDa. So far, there have been no reports of hexameric structure of PPi-PFK, but the *R. rubrum* PPi-PFK was shown to be present as monomer, dimer, or octamer due to association–dissociation [24].

The *Mb. nodulans* PPi-PFK catalyzed F6P phosphorylation and FBP dephosphorylation most actively at pH 7.5 and 30°C (A_{max} = 87 and 75 U/mg protein, respectively). The dependence of the enzyme activity on concentrations of the substrates of the forward and reverse reactions corresponded to Michaelis–Menten kinetics. The following apparent K_m values were obtained for F6P, FBP, PPi, and Pi: 0.65 ± 0.11, 0.043 ± 0.001, 0.006 ± 0.001, and 1.04 ± 0.10 mM, respectively.

The activity of the *Ms. trichosporium* PPi-PFK was maximal at 50°C and pH 7.5, being practically equal in the forward and reverse reactions (88 and 89 U/mg protein). The dependence of PPi-PFK activity on concentrations of the substrates also had hyperbolic character. For F6P, FBP, PPi, and Pi the following apparent K_m values at 30°C were obtained: 0.24 ± 0.04, 0.074 ± 0.008, 0.011 ± 0.006, and 2.4 ± 0.2 mM, respectively.

For *Mb. nodulans* PPi-PFK the apparent K_m values for Mg^{2+} were 0.047 ± 0.001 and 0.36 ± 0.01 mM for the forward and reverse reactions, respectively. Similar apparent K_m values for Mg^{2+} were calculated for the *Ms. trichosporium* enzyme (0.030 ± 0.001 and 0.363 ± 0.007 mM). At 1 mM concentration Co^{2+} and Mn^{2+} ions could replace Mg^{2+} (~50 and 65% of Mg^{2+} activity, respectively) in the reactions catalyzed by the *Ms. trichosporium* PPi-PFK. The activity of the *Mb. nodulans* enzyme with Mn^{2+} was 81% of its activity with Mg^{2+}.

No effectors were revealed for the *Mb. nodulans* PPi-PFK, similarly to most bacterial PPi-PFKs (see "Materials and Methods"). Therefore, we assume that the enzyme activity in the glycolytic and gluconeogenic directions is regulated by the substrate and product concentrations. In contrast, 1 mM AMP or ADP inhibited the activity of the *Ms. trichosporium* enzyme in the forward reaction by 90 and 50%, and completely in the reverse reaction. Among previously characterized bacterial PPi-PFKs, only the *R. rubrum* enzyme was inhibited by AMP, ADP, and, to a lesser extent, by ATP [24]. It is reasonable to assume that in *Ms. trichosporium* the synthesis and cleavage of C_6-compounds depend on the cell energy status and are regulated at the level of PPi-PFK activity.

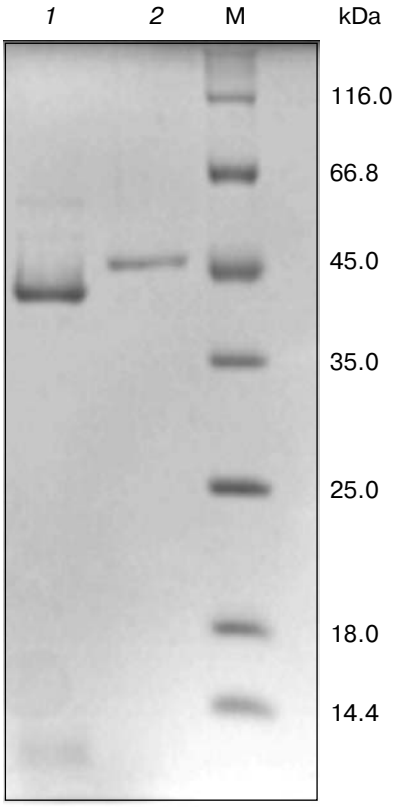

Fig. 1. Electrophoresis of *Mb. nodulans* (*1*) and *Ms. trichosporium* (*2*) PPi-PFK in denaturing conditions. M, marker proteins.

Like other PPi-PFKs, the enzymes from *Mb. nodulans* and *Ms. trichosporium* were highly specific to PPi as the phosphoryl donor. Glucose-6-phosphate, glucose-1-phosphate, fructose-1-phosphate, mannose-6-phosphate, ribose-5-phosphate, ribose-1-phosphate, and erythrose-4-phosphate were not effective as the phosphoryl acceptors for the enzymes from both bacteria. However, PPi-PFK from *Ms. trichosporium* displayed ability to phosphorylate ribulose-5-phosphate, but with low activity (1.4% of the F6P activity). In contrast, the *Mb. nodulans* PPi-PFK had no corresponding activity. Low activity with ribulose-5-phosphate was earlier reported for PPi-PFK from *Mc. capsulatus* Bath [11].

Table 3. Specificity of PPi-PFK to C_6- and C_7-phosphosugars

Substrate	*Mb. nodulans*			*Ms. trichosporium*		
	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $mM^{-1}\cdot sec^{-1}$	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $mM^{-1}\cdot sec^{-1}$
F6P	0.65	16	24	0.24	24	99
S7P	0.21	8.3	40	0.51	23	45
FBP	0.043	13.4	312	0.070	24	343

Table 4. Some characteristics of PPi-PFK from methylotrophic bacteria

Substrate	*Mm. alcaliphilum* 20Z [12]	*M. methanica* [10]	*Mc. capsulatus* Bath [11]	*Mb. nodulans* ORS 2060	*Ms. trichosporium* OB3b
	A_{max}, U/mg protein				
F6P	577	840	7.6	87	88
FBP	805	850	7.6	75	89
S7P	0.18	n.d.	31	47	85
	K_m, mM				
PP_i	0.118	0.051	0.027	0.006	0.011
F6P	0.64	0.39	2.27	0.65	0.24
S7P	1.01	n.d.	0.030	0.21	0.51
P_i	3.4	1.7	8.69	1.04	2.4
FBP	0.095	0.1	0.328	0.043	0.07
Mg^{2+} *	0.22	0.038	0.028	0.047	0.030
Mg^{2+} **	0.33	0.35	2	0.32	0.36
M, kDa (number of subunits)	180 (4)	92 (2)	90 (2)	180 (4)	270 (6)
pH-Optimum	7.5	8.0	7.0	7.5	7.5
T_{opt}, °C	30	40	30	30	50
Effectors	–	–	–	–	ADP, AMP

Notes: –, not found; n.d., not determined; M, molecular mass; T_{opt}, temperature optimum.
* Forward reaction.
** Reverse reaction.

As demonstrated, PPi-PFK from a number microorganisms can phosphorylate S7P, but with lower activity and specificity as compared with F6P [25, 26]. The enzyme from *Mb. nodulans* phosphorylated S7P with two times lower activity (A_{max} = 47 U/mg protein) as compared with F6P, but the apparent K_m value for the C_7-substrate was lower (0.21 ± 0.02 mM). Judging from the specificity constant k_{cat}/K_m, PPi-PFK from *Mb. nodulans* prefers S7P to F6P (Table 3). The activities of the *Ms. trichosporium* PPi-PFK with S7P and F6P were approximately equal (85 and 89 U/mg protein), although the apparent K_m value was higher for S7P (0.51 ± 0.26 mM) than for F6P (Table 4). However, the best substrate for the enzymes from both methylotrophs was FBP (Tables 3 and 4), which was in agreement with the functioning of the serine pathway in these bacteria and the

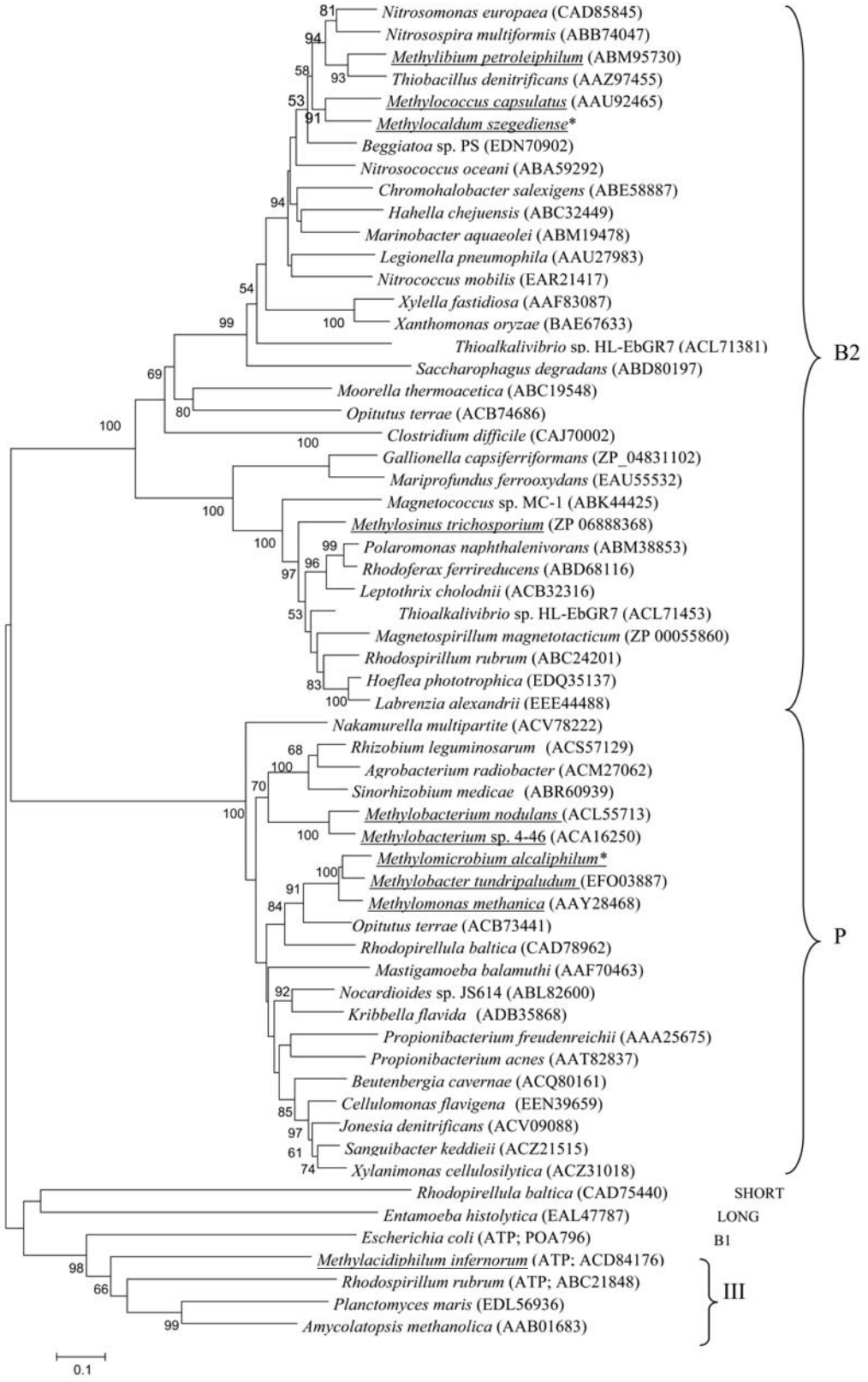

Fig. 2. Phylogenetic tree based on deduced amino acid sequences of PFK from methylotrophs (underlined) and other bacteria. Phosphate donor (ATP) for ATP-dependent PFK and accession numbers of sequences in GenBank are indicated in parentheses. Monophyletic groups are named according to the accepted classification [1, 29]. * The *pfp* gene sequences were obtained from the partially completed genome sequences of *Mm. alcaliphilum* 20Z and *Methylocaldum szegediense* O-12.

necessity of synthesis of C_6-phosphosugar from C_3-compounds.

Relatively high activity of the *Mb. nodulans* PPi-PFK with S7P may be due to the ability of this facultative methylotroph to grow on some C_5- and C_6-sugars, but not on glucose [27]. Considering this, PPi-PFK can participate in the reactions of the pentose phosphate pathway. Moreover, the specific activity of PPi-PFK in extracts of *Mb. nodulans* cells growing on L-arabinose was twofold higher than that of the methanol-grown cells (50 and 22 mU/mg protein, respectively).

It has been shown for PPi-PFK from *Entamoeba histolytica* that the enzyme has only two times lower apparent K_m for S7P than that for F6P [28]. For this amitochondrial protist having no transaldolase and sedoheptulose-1,7-bisphosphatase (SBPase), a modified version of the non-oxidative pentose phosphate pathway with the participation of PPi-PFK has been proposed [28, 29]. The high activity with S7P and preference for this substrate (the apparent K_m for S7P was 1000 times lower than for F6P (Table 4)) was shown for PPi-PFK from the methanotroph *Mc. capsulatus* Bath. This implies that the enzyme may participate in the reductive segment of the Calvin cycle, fulfilling the function of SBPase [11, 13]. In this connection, it should be noted the presence in the *Mb. nodulans* genome of two genes coding the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (accession numbers ACL58349 and ACL57391) and a gene of a putative phosphoribulokinase (PRK) (ACL55364). Although in the genome of *Ms. trichosporium* OB3b the gene for PRK (accession number EFH01572) is present, no open reading frame for the large or small subunits of RuBisCO was found. And the PPi-PFKs from *Mm. alcaliphilum* 20Z and *M. methanica* 12, which have no genes for the Calvin cycle enzymes, showed only negligible activity with S7P [12]. Thus, although the ability to phosphorylate S7P is common to all PPi-PFKs, the enzyme from methylotrophic bacteria displays certain "specialization" to different types of C_1-metabolism.

Phylogenetic analysis showed that the PPi-PFKs from *Mb. nodulans* ORS 2060 and *Ms. trichosporium* OB3b display only 17% identity of translated amino acid sequences and belong to different subgroups of type II 6-phosphofructokinases according to the classification of Bapteste et al. [1] (Fig. 2). The *Ms. trichosporium* PPi-PFK is a member of the B2 subgroup and has the highest similarity to the enzymes from α- and β-proteobacteria: *Polaromonas naphthalenivorans* (77% identity), *Rhodoferax ferrireducens* (76%), and *R. rubrum* (75%). PPi-PFK from the γ-proteobacterium *Mc. capsulatus* Bath also belongs to this subgroup (34% identity with the *Ms. trichosporium* enzyme). The *Mb. nodulans* PPi-PFK as well as the enzymes from the RuMP-pathway methanotrophs *Mm. alcaliphilum* 20Z, *M. methanica* 12, and *Methylobacter tundripaludum* belong to the P subgroup of PPi-PFKs. Remarkably, the latter subgroup includes enzymes from anaerobes for which predominantly glycolytic function has been shown.

The occurrence of PPi-PFK in microorganisms is usually associated with anaerobic type of metabolism. Aerobic methanotrophs, including *Ms. trichosporium* OB3b, are microaerophiles being able to survive for a long period in an oxygen-free environment due to fermentation of endogenous substrates [30], the reutilization of which by means of PPi-PFK may play an important role. *Methylobacterium nodulans* ORS 2060 is a rhizospheric phytosymbiont possessing in its life cycle an anaerobic bacteroid stage, which realizes in the nodules of leguminous plants the energy-requiring process of nitrogen fixation [31]. In this case, glycolysis where PPi instead of ATP is consumed in one reaction is a way of effectively drawing energy from plant biosynthesis products. It should be noted that the *Mb. nodulans* PPi-PFK is closest in amino acid sequence (64-65% identity) to the enzymes from *Methylobacterium* sp. 4-46, *Rhizobium leguminosarum*, *Sinorhizobium medicae*, and *Agrobacterium radiobacter* (Fig. 2), also stimulating nodulation in higher plants. However, the *pfp* genes were not found in the genomes of phyllosphere symbionts of the *Methylobacterium* genus, highlighting the relationship of the presence of PPi-PFK with the ability of some bacteria to live under anaerobic conditions of phytosymbiosis, which is consistent with a significant role of lateral transfer of the *pfp* gene in microorganisms [1].

The data provided in Table 4 indicate that the PPi-PFKs of methylotrophs prefer FBP as a substrate and, therefore, functions predominantly in the gluconeogenic direction. This reaction can create a pool of PPi, and its reutilization with the participation of the membrane H^+-translocating pyrophosphatase can lead to ATP synthesis bypassing the respiratory chain. This correlates with the extraordinarily low activity of the soluble inorganic pyrophosphatase in methanotrophs possessing PPi-PFK [6] and with the presence of H^+-pyrophosphatase genes in the genomes of these bacteria.

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REFERENCES

1. Bapteste, E., Moreira, D., and Philippe, H. (2003) *Gene*, **318**, 185-191.
2. Siebers, B., Klenk, H. P., and Hensel, R. (1998) *J. Bacteriol.*, **180**, 2137-2143.
3. Chi, A., and Kemp, R. G. (2000) *J. Biol. Chem.*, **275**, 35677-35679.
4. Trotsenko, Y. A., and Shishkina, V. N. (1990) *FEMS Microbiol. Lett.*, **87**, 267-271.
5. Alves, A. M. C. R., Euverink, G. J. W., Hektor, H. J., Hessels, G. I., van der Vlag, J., Vrijbloed, J. W.,

Hondmann, D., Visser, J., and Dijkhuizen, L. (1994) *J. Bacteriol.*, **176**, 6827-6835.
6. Beschastny, A. P., Khmelenina, V. N., Rozova, O. N., and Trotsenko, Y. A. (2008) *Mikrobiologiya*, **77**, 591-594.
7. Taylor, S. C., Dalton, H., and Dow, C. S. (1981) *J. Gen. Microbiol.*, **122**, 89-94.
8. Eshinimaev, B. T., Medvedkova, K. A., Khmelenina, V. N., Suzina, N. E., Osipov, G. A., Lysenko, A. M., and Trotsenko, Y. A. (2004) *Mikrobiologiya*, **73**, 530-539.
9. Trotsenko, Y. A., and Murrell, J. C. (2008) *Adv. Appl. Microbiol.*, **63**, 183-229.
10. Beschastny, A. P., Sokolov, A. P., Khmelenina, V. N., and Trotsenko, Y. A. (1992) *Biokhimiya*, **57**, 1215-1221.
11. Reshetnikov, A. S., Rozova, O. N., Khmelenina, V. N., Mustakhimov, I. I., Beschastny, A. P., Murrell, J., and Trotsenko, Y. A. (2008) *FEMS Microbiol. Lett.*, **288**, 202-210.
12. Rozova, O. N., Khmelenina, V. N., Vuilleumier, S., and Trotsenko, Y. (2010) *Res. Microbiol.*, **161**, 861-868.
13. Khmelenina, V. N., Rozova, O. N., and Trotsenko, Y. A. (2011) *Meth. Enzymol.*, **495**, 1-14.
14. Khmelenina, V. N., Beschastny, A. P., Gayazov, R. R., and Trotsenko, Y. A. (1994) *Mikrobiologiya*, **63**, 95-98.
15. Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., and Dreyfus, B. (2001) *J. Bacteriol.*, **183**, 214-220.
16. Kalyuzhnaya, M., Khmelenina, V. N., Kotelnikova, S., Holmquist, L., Pedersen, K., and Trotsenko, Y. A. (1999) *Syst. Appl. Microbiol.*, **22**, 565-572.
17. Baker, R. T., Catanzariti, A.-M., Karunasekara, Y., Soboleva, T. A., Sharwood, R., Whitney, S., and Board, P. (2005) *Meth. Enzymol.*, **398**, 540-554.
18. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
19. Slater, G. G. (1969) *Anal. Chem.*, **41**, 1039-1041.
20. Shacterle, G. R., and Pollack, R. L. (1973) *Anal. Biochem.*, **51**, 654-657.
21. Thomson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.*, **24**, 4876-4882.
22. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) *Molecular Biology and Evolution*, 10.1093/molbev/msm092.
23. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: a Laboratory Manua*l, 3rd Edn., Cold Spring Harbor Laboratory, N.-Y.
24. Pfleiderer, C., and Klemme, J. H. (1980) *Z. Naturforsch.*, **35**, 229-238.
25. Bertagnolli, B. L., Younathan, E. S., Voll, R. J., Pittman, C. E., and Cook, P. F. (1986) *Biochemistry*, **25**, 4674-4681.
26. Mertens, E., van Schaftingen, E., and Muller, M. (1989) *Mol. Biochem. Parasitol.*, **37**, 183-190.
27. Jourand, P., Giraud, E., Bena, G., Sy, A., Willems, A., Gillis, M., Dreyfus, B., and de Lajudie, P. (2004) *Int. J. System. Evol. Microbiol.*, **54**, 2269-2273.
28. Susskind, B. M., Warren, L. G., and Reeves, R. E. (1982) *Biochem. J.*, **204**, 191-196.
29. Loftus, B., Anderson, I., Davies, R., Alsmark, U. C. M., Samuelson, J., Amedeo, P., Roncaglia, P., Berriman, M., Hirt, R. P., Mann, B. J., Nozaki, T., Suh, B., Pop, M., Duchene, M., Ackers, J., Tannich, E., Leippe, M., Hofer, M., Bruchhaus, I., Willhoeft, U., Bhattacharya, A., Chillingworth, T., Churcher, C., Hance, Z., Harris, B., Harris, D., Jagels, K., Moule, S., Mungall, K., Ormond, D., Squares, R., Whitehead, S., Quail, M. A., Rabbinowitsch, E., Norbertczak, H., Price, C., Wang, Z., Guillen, N., Gilchrist, C., Stroup, S. E., Bhattacharya, S., Lohia, A., Foster, P. G., Sicheritz-Ponten, T., Weber, C., Singh, U., Mukherjee, C., El-Sayed, N. M., Petri, W. A., Jr., Clark, C. G., Embley, T. M., Barrell, B., Fraser, C. M., and Hall, N. (2005) *Nature*, **433**, 865-868.
30. Muller, M., Lee, J. A., Gordon, P., Gaasterland, T., and Sensen, C. W. (2001) *J. Bacteriol.*, **183**, 6714-6716.
31. Roslev, P., and King, G. M. (1995) *Appl. Environ. Microbiol.*, **61**, 1563-1570.