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Characterization of Recombinant Fructose-1,6-Bisphosphate Aldolase from *Methylococcus capsulatus* Bath

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Abstract—The gene *fba* from the thermotolerant obligate methanotroph *Methylococcus capsulatus* Bath was cloned and expressed in *Escherichia coli* BL21(DE3). The fructose-1,6-bisphosphate aldolase (FBA) carrying six His on the C-end was purified by affinity metal chelating chromatography. The *Mc. capsulatus* FBA is a hexameric enzyme (240 kDa) that is activated by Co^{2+} and inhibited by EDTA. The enzyme displays low K_m to fructose-1,6-bisphosphate (FBP) and higher K_m to the substrates of aldol condensation, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The FBA also catalyzes sedoheptulose-1,7-bisphosphate cleavage. The presence of Co^{2+} in the reaction mixture changes the kinetics of FBP hydrolysis and is accompanied by inhibition of the reaction by 2 mM FBP. Phylogenetically, the *Mc. capsulatus* enzyme belongs to the type B of class II FBAs showing high identity of translated amino acid sequence with FBAs from autotrophic bacteria. The role of the FBA in metabolism of *Mc. capsulatus* Bath, which realizes simultaneously three C_1 assimilating pathways (the ribulose monophosphate, the ribulose bisphosphate, and the serine cycles), is discussed.

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Fructose-1,6-bisphosphate aldolase (FBA, EC 4.1.2.13) catalyzing the reversible cleavage of fructose-1,6-bisphosphate (FBP) into triose phosphates fulfils in cells an amphibolic function operating in the catabolic Embden–Meyerhof–Parnas (EMP) pathway and in anabolic pathways—gluconeogenesis and the Calvin cycle. Two classes of the FBAs significantly differing in the reaction mechanisms are known [1]. The FBAs of class I make a Schiff-base between the carbonyl substrate FBP or dihydroxyacetone phosphate (DHAP) and the ε-amino group of a lysine residue in the active site, being inactivated by borohydride ($NaBH_4$) and independent of divalent metals ions. The FBAs of class II are metal-dependent enzymes and are inhibited by EDTA. The enzymes of the two classes show no homology in amino acid sequences, thus aldol function twice and independently appeared in evolution [2, 3]. Distribution of two FBA classes among organisms of different taxonomic groups has no clear regularity and explanation so far.

According to the amino acid sequences, class II FBAs are divided into two different groups referred to as type A and type B [4]. Analysis of the annotated genome of the thermotolerant methanotroph *Methylococcus capsulatus* Bath has revealed the presence of two identical copies of the *fba* gene [5]. As known, this methanotroph utilizes methane carbon at the level of formaldehyde and CO_2 by reactions of three cyclic biochemical pathways [6]. In the major ribulose monophosphate (RuMP) cycle, the first products of condensation of formaldehyde and ribulose-5-phosphate are hexose phosphates. Alternatively, in the minor serine pathway and in the Calvin cycle the respective primary products are three-carbon compounds—serine or 3-phosphoglycerate. Moreover, in *Mc. capsulatus* Bath the C_6-phosphosugars synthesized in the RuMP cycle are cleaved into pyruvate and glyceralde-

Abbreviations: DHAP, dihydroxyacetone phosphate; EMP, Embden–Meyerhof–Parnas; FBA, fructose-1,6-bisphosphate aldolase; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; PP_i-PFK, PP_i-dependent phosphofructokinase; RuBP, ribulose-1,5-bisphosphate; RuMP, ribulose monophosphate; SBP, sedoheptulose-1,7-bisphosphate.

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hyde phosphate via the Entner–Doudoroff pathway. Hence, both glycolytic and gluconeogenic substrates (C_6- and C_3-compounds, respectively) can be formed bypassing the EMP pathway. In view of the abovementioned metabolic features of *Mc. capsulatus* Bath, it was of interest to study the properties of FBA from this methanotroph and to follow evolution relationships of FBA from bacteria of different taxonomic groups.

MATERIALS AND METHODS

Bacteria and growth conditions. *Methylococcus capsulatus* Bath was grown under methane–air atmosphere (1 : 1) in mineral medium P at 37°C [7]. *Escherichia coli* strain BL21(DE3) was grown in selective LB (Luria–Bertani) broth or agar [8] at 37°C.

FBA cloning, expression, and purification. The chromosomal DNA from *Mc. capsulatus* cells was prepared as previously described [9]. The *fba* gene (MCA3041) was amplified by PCR using primers designed from the sequence available in GenBank (accession number AAU90900) [5]: forward (5′-TTATCTCCATATGGCTTTGATCTCCCTGC) and reverse (5′-TTAAGCTTGCGGACGATCGGATCGAGT) containing recognition sites for endonuclease restriction *Nde*I and *Hind*III, respectively. The PCR-product was purified on a Wizard column (Promega, USA), incubated with the site-specific endonucleases *Nde*I and *Hind*III, and ligated in the expression vector pET22b$^+$ treated with these endonucleases. The resulting plasmid pET*fba* was transformed into *E. coli* strain BL21(DE3). 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 cultivated until $A_{600} = 0.6$-0.7. The protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at final concentration 1 mM. After incubation at 37°C for 5 h, the cells were harvested by centrifugation at 6000*g* for 20 min (4°C) and stored at –20°C. The FBA-His_6-tag was purified by affinity chromatography on a Ni^{2+}-NTA column as earlier described [10] and analyzed by 12% SDS-PAGE [11].

Determination of FBA molecular mass. The quaternary structure of the enzyme was determined by nondenaturing gel electrophoresis and gel filtration. The gel electrophoresis was carried out using pore-limiting gradient polyacrylamide (4-30%) [12] and protein markers (Pharmacia, USA) containing thyroglobulin (670 kDa, dimer), ferritin (440 kDa, 24 subunits), catalase (232 kDa, tetramer), lactate dehydrogenase (140 kDa, tetramer), and bovine serum albumin (67 kDa, monomer). The protein was gel filtered on calibrated Sephacryl S-200 column equilibrated with 20 mM Tris-HCl, pH 7.2, containing 100 mM NaCl. The marker proteins were thyroglobulin (670 kDa, dimer), bovine γ-globulin (158 kDa, dimer), chicken ovalbumin (44 kDa, monomer), equine myoglobin (17 kDa, monomer), and vitamin B_{12} (1.35 kDa) (BioRad, USA).

FBA activity assay. Activity of the FBA towards the FBP or sedoheptulose-1,7-bisphosphate (SBP) cleavage was assayed at 30°C by spectrophotometrically measuring NADH oxidation at 340 nm with the coupling enzyme α-glycerol-3-phosphate dehydrogenase (Sigma, USA). The reaction mixture (1 ml) containing 50 mM Tris-HCl buffer (pH 7.5), 0.25 mM NADH, 5 U α-glycerol-3-phosphate dehydrogenase, and up to ~10 mU purified FBA was preheated for 4 min. The reaction was initiated by addition of 4 mM substrate (FBP or SBP). NADH oxidation was monitored on a Shimadzu UV-1700 spectrophotometer (Japan). Kinetic parameters (A_{max} and K_m) were determined by measuring FBA activity with different concentrations of FBP or SBP and calculated using the Enzyme Kinetics Module of the SigmaPlot 10 program. The kinetic constants for FBP were obtained both in the presence and absence of 20 μM Co^{2+} in the reaction mixture. The effect of pH on the FBA activity was tested using the following buffers (50 mM): acetate (pH 5.0-5.5), phosphate (pH 6.0-6.5), Tris-HCl (pH 7.0-9.0), and glycine-NaOH (pH 9.5-10.0). To determine the thermal stability of FBA, aliquots of the enzyme were incubated in Eppendorf tubes at 30, 40, 50, 60, and 80°C from 10 min to 2 h and rapidly cooled on ice, and then the residual enzyme activity was determined at 30°C as described above. The residual activity was calculated by comparison with activity of the non-heated enzyme. The effect of Zn^{2+}, Co^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, and Mg^{2+} (1 mM) on the FBA activity was determined by the standard method. The effect of EDTA was estimated by two procedures: EDTA was added directly before the reaction was started by FBP addition or the enzyme was preheated in 50 mM Tris-HCl (pH 7.5) with 1 mM EDTA at 50°C for 10 min followed by measurement of the residual FBA activity.

The FBA activity in the direction of FBP synthesis was estimated spectrophotometrically with coupling enzymes, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase (from rabbit muscle; Sigma), and recombinant PP_i-dependent phosphofructokinase (PP_i-PFK) from *Methylomonas methanica* 12 obtained by cloning and expression of the *pfk* gene (AAY28468) and purified as described [13]. The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 2 mM DHAP, 2 mM glyceraldehyde-3-phosphate (GAP), 1 mM $NADP^+$, 1 mM NaH_2PO_4, 4 mM $MgCl_2$, 5 U of each coupling enzyme, and ~15 mU purified FBA. The reaction was initiated by addition of various concentrations of DHAP or GAP, and $NADP^+$ reduction was registered at 340 nm. K_m and A_{max} values were calculated using the Enzyme Kinetics Module of the SigmaPlot 10 program.

DNA manipulations. Methods for plasmid isolation, restriction, agarose gel electrophoresis, ligation, and transformation of *E. coli* cells were performed according

to Sambrook and Russell [14]. Restriction enzymes, T4 DNA-ligase, *Taq* DNA-polymerase, and a dNTP mixture were produced by Fermentas (Lithuania).

Sequence analyses. Sequences from the NCBI protein database (http://www.ncbi.nlm.nih.gov) were obtained using the BLAST program. Sequence of the *Methylomicrobium alcaliphilum fba* gene was obtained from the partially completed genome (http://www.genoscope.cns.fr/externe/English/corps_anglais.html). Sequence alignments were generated with the CLUSTAL X program (version 1.8) [15]. Minor corrections in alignment were made manually. The dendrogram was constructed using the MEGA 4 program [16].

RESULTS

Cloning of *fba* gene and purification of FBA. The putative *fba* gene of *Mc. capsulatus* Bath was isolated from the genomic DNA by PCR. The PCR product was cloned into the bacterial expression vector pET22b(+) designed for expression of a C-terminal His_6-tagged fusion protein under control of the T7 promoter. The recombinant plasmid was transformed into *E. coli* BL21(DE3) cells, and protein expression was induced by IPTG addition in the early-log phase of culture. The His_6-tagged FBA from crude *E. coli* extracts was purified by a single step by affinity chromatography. SDS-PAGE of the purified FBA-His_6 showed a single band of ~40 kDa (Fig. 1), which is in good agreement with the molecular mass calculated from the predicted amino acid sequence (38.5 kDa).

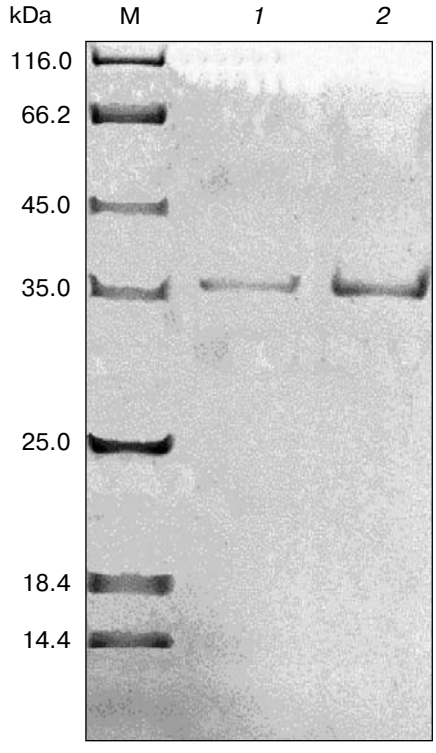

Fig. 1. 12% SDS-PAGE of recombinant FBA. M, molecular mass markers; *1*, *2*) 2 and 5 μg protein, respectively.

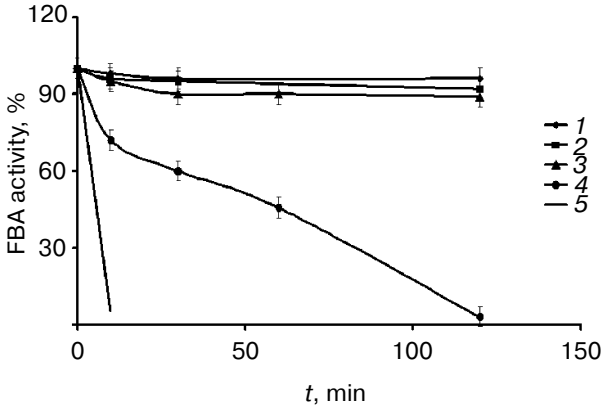

Fig. 2. Residual activity of FBA at 30, 40, 50, 60, and 80°C (*1-5*, respectively).

Characterization of recombinant FBA. According to gel filtration on Sephacryl S-200, the molecular mass of the FBA was 240 kDa, which corresponded to the hexameric enzyme form. Native gradient PAGE revealed a single band at electrophoretic mobility similar to that of catalase (240 kDa, tetramer).

The FBA was active in the pH range 5.5-8.5 and increased by 25% at pH 9.0-9.5. Thus, the *Mc. capsulatus* enzyme had more alkaline pH optimum than those of class II FBAs (Table 1). The enzyme activity increased 2.5 times in the presence of Co^{2+}. However, Mg^{2+} or Ca^{2+} had no effect, whereas Zn^{2+}, Cu^{2+}, and Cd^{2+} at concentration 1 mM lowered activity by 60, 80, and 90%, respectively. Incubation of the FBA with 1 mM EDTA completely inhibited the enzyme activity. This suggested that the *Mc. capsulatus* Bath enzyme belongs to the class II FBAs. The enzyme activity was retained after incubation for 2 h at 30, 40, or 50°C. The half-life of the enzyme was 1 h at 60°C, and the enzyme was fully inactivated after heating at 80°C for 10 min (Fig. 2). It should be noted that thermal stability of the FBA correlated with the physiology of *Mc. capsulatus* Bath, which is capable of growth at 37-55°C.

Kinetic properties. The initial rate of the aldol FBP cleavage was determined at various concentrations of the substrate. In the absence of Co^{2+} in the reaction mixture, the dependence of reaction velocity on FBP concentration exhibited Michaelis–Menten kinetics (Fig. 3a). The apparent K_m value of the enzyme was 0.018 mM (A_{max} = 1.26 U/mg protein). However, addition of 20 μM Co^{2+} resulted in appearance of two K_m values: 0.0052 and 1.1 mM ($A_{max} = 4.46 \pm 0.11$ and 13.9 ± 2.8 U/mg protein) at substrate concentrations of 0.008-0.5 and 0.5-1.5 mM,

Table 1. Some properties of FBA II from different microorganisms

Microorganism	A_{max}, U/mg protein		K_m, mM		Ion	M_r, kDa (subunit number)	pH optimum
	FBP	SBP	FBP	SBP			
Methylococcus capsulatus (type B)	13.9	0.7	0.005/1.1*	0.19	Co^{2+}	240 (6·38.5)	9.0-9.5
Anoxybacillus gonensis (type B) [17]	2.4	–	0.576	–	Zn^{2+}	(·31.9)	8.5
Th. aquaticus (type B) [18]	278	–	0.01/3.17**	–	Co^{2+}	165 (4·34)	6.5
Synechocystis sp. (type B) [19]	5.9	–	0.008	0.008	Co^{2+}	312 (8·39)	7.0-7.5
Euglena gracilis (type A) [3]	22	–	0.175	–	Mn^{2+}, Co^{2+}, Cu^{2+}	80 (2·38.5)	7.5

Note: –, not determined.

* K_m values were determined in the presence of 20 μM Co^{2+} in the reaction mixture at FBP concentrations 0.008-0.5 mM (numerator) and 0.5-1.5 mM (denominator).

** K_m values were determined in the presence of 100 μM Co^{2+} in the reaction mixture at FBP concentrations 0.01-2 mM (numerator) and 2-10 mM (denominator).

respectively (Fig. 3b). FBP at concentration >2 mM inhibited the FBA activity in the presence of 20 μM Co^{2+} (Fig. 3, a and b) but not in the absence of this metal. FBA also catalyzed SBP cleavage (apparent K_m = 0.19 mM, A_{max} = 0.71 U/mg protein). This enzyme activity was stimulated twofold in the presence of 20 μM Co^{2+}, but no inhibition by high SBP concentrations (2-4 mM) was observed either in the presence or in the absence of this ion in the reaction mixture.

In the reverse reaction, the dependence of reaction velocity on the substrate concentrations had hyperbolic form (data not shown). Apparent K_m values for DHAP and GAP were 0.095 and 1.1 mM, respectively. Maximal reverse reaction activity of the FBA was 0.46 U/mg protein (Table 2). Importantly, the condensation reaction of GAP and DHAP has been rarely studied for FBAs due to complexity of FBP detection. This problem was resolved by use of PP_i-PFK catalyzing the reversible reaction of fructose-6-phosphate formation from FBP as the coupling enzyme. We obtained a highly active preparation of this enzyme by cloning of the *pfk* gene from the obligate methanotroph *Methylomonas methanica* [10, 13].

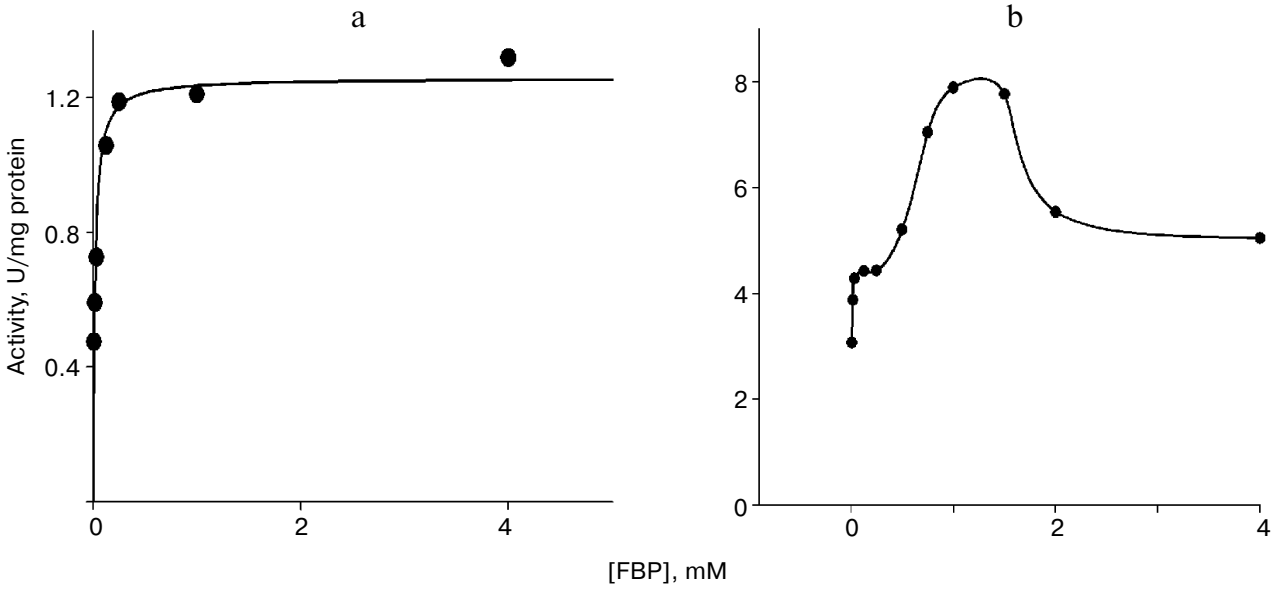

Fig. 3. Dependence of *Mc. capsulatus* Bath FBA activity on FBP concentration as a substrate in the absence of Co^{2+} (a) and in the presence of 20 μM Co^{2+} (b).

Table 2. Kinetic properties for FBA from *Mc. capsulatus* Bath

Substrate	K_m, mM	A_{max}, U/mg protein	k_{cat}, min^{-1}
FBP	0.018 ± 0.002	1.26 ± 0.04	0.302 ± 0.009
FBP (0.008-0.5 mM) + 20 μM Co^{2+}	0.0052 ± 0.0008	4.46 ± 0.11	1.07 ± 0.03
FBP (0.5-1.5 mM) + 20 μM Co^{2+}	1.1 ± 0.4	13.9 ± 2.8	3.3 ± 0.7
SBP	0.19 ± 0.05	0.71 ± 0.04	0.170 ± 0.009
DHAP	0.095 ± 0.027	0.49 ± 0.03	0.118 ± 0.007
GAP	1.17 ± 0.12	0.46 ± 0.02	0.110 ± 0.005

Phylogeny of FBAs. To construct the phylogenetic tree, the MEGA 4 program [16] was used. Comparative analysis of the translated amino acid sequences of putative *fba* genes from presented bacterial genomes database showed that *Mc. capsulatus* Bath FBA is most closely related to FBA of the obligate chemolithotrophic bacterium *Thiobacillus denitrificans* (80% identity). Other close relatives of the *Mc. capsulatus* Bath FBA are those from the facultative autotrophs *Magnetospirillum magnetotacticum* (72%), *Xanthobacter autotrophicus*, *X. flavus*, and *Nitrosomonas europaea* (71%), as well as from methylotrophic bacteria *Methylibium petroleiphilum* (71%), *Methylobacillus flagellatus* (68%), and *Methylomicrobium alcaliphilum* (60%) (Fig. 4). The *Mc. capsulatus* Bath FBA has low identity with FBAs from other methanotrophic bacteria—thermoacidophilic *Methylacidiphilum infernorum* V4 (36%) and acidotolerant *Methylocella silvestris* (30%). It should be noted that FBAs from these autotrophs and methylotrophs constitute the type B of class II FBAs and have low homology with type A of class II FBAs [4]. So, identity of the amino acid sequences between the *Mc. capsulatus* Bath FBA and the *E. coli* enzyme belonging to type A of class II FBAs was only about 20%.

DISCUSSION

Our data suggest that the FBA from thermotolerant methanotroph *Mc. capsulatus* Bath belongs to class II FBAs since it is activated by Co^{2+} and inhibited by EDTA. The enzyme in the native state is a homohexamer with molecular mass of 240 kDa. Among the class II FBAs, dimeric, tetrameric, and octameric forms of the enzyme have been described earlier, the latter form exists, for example, in *Synechocystis* sp. [19]. However, hexameric oligomerization of this class of FBAs was not reported. Usually, class II aldolases have a peak of activity in the pH range from 7.0 to 9.0 [20] or at pH 6.5 for *Thermus aquaticus* FBA [18]. The maximal activity of the *Mc. capsulatus* Bath FBA is displaced to more alkaline values than previously reported for this enzyme. Addition of Co^{2+} (20 μM) increases FBA activity, decreases K_m for FBP, and provokes inhibition by 2 mM FBP. Possibly, Co^{2+} changes aggregation state of the enzyme subunits, thus influencing the kinetic constants. Complicated kinetics of FBA displaying two K_m for FBP was revealed for the enzyme from hyperthermophilic bacterium *Thermus aquaticus* (Table 1), but the effect of Co^{2+} on the enzyme behavior was not studied.

Based on comparison of amino acid sequences, FBAs of class II have been grouped into types A and B [4]. The enzyme from *Mc. capsulatus* Bath belongs to type B of FBAs (Fig. 4). The closest relatives of *Mc. capsulatus* Bath FBA are the enzymes from autotrophic bacteria where the *fba* genes are located in operons coding for the Calvin cycle enzymes and denoted as *cbbA* genes (for example, in *T. denitrificans*) [21]. Moreover, it was found that in *X. flavus*, the FBA (type B, class II) operates in the reductive pentose phosphate pathway of the Calvin cycle, and it is not involved in gluconeogenesis or glycolysis [22].

In the genome of *Mc. capsulatus* Bath, two identical copies of the *fba* gene exist in a direct repeat composed of the genes encoding transketolase, a hypothetical protein, hexulose phosphate synthase, and hexulose phosphate isomerase separated by a transaldolase gene [5]. This repeat is located between the gene coding for a putative transcriptional regulator of the ribulose bisphosphate carboxylase and the gene for the phosphoribulokinase, thus additionally indicating that the *Mc. capsulatus* Bath FBA is involved in the Calvin cycle. Moreover, the *Mc. capsulatus* FBA catalyzes SBP cleavage, which is an important reaction of Calvin cycle rearrangements. The putative role of the enzyme in the Calvin cycle correlates with unique metabolic feature of *Mc. capsulatus* Bath, which is able to synthesize both C_6- and C_3-compounds in the first stages of methane assimilation at the oxidation levels of formaldehyde and CO_2 bypassing the EMP pathway.

Remarkably, the other relatives of *Mc. capsulatus* Bath FBA are those of obligate methylotrophs with the

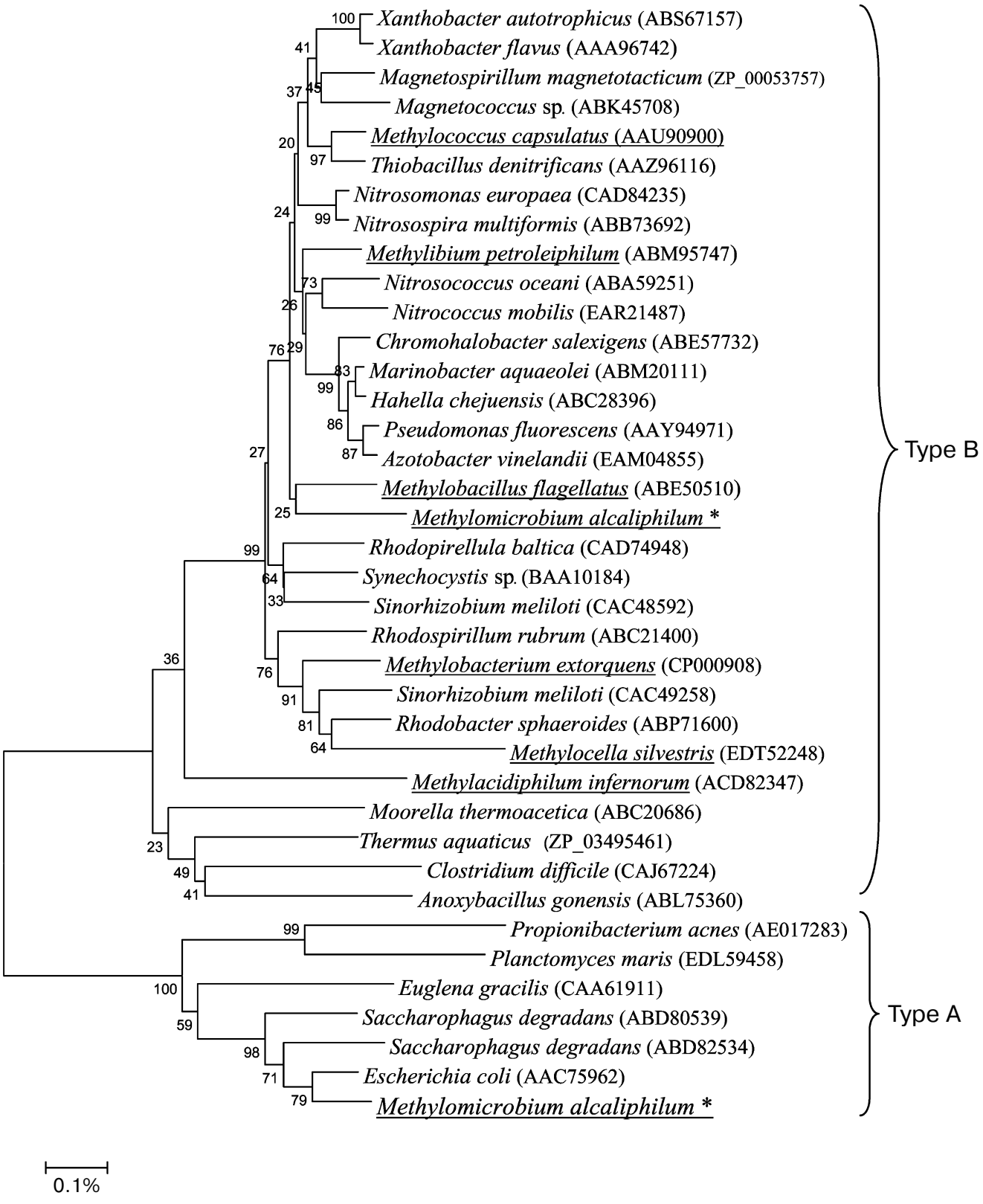

Fig. 4. Phylogenetic tree of some class II FBAs. Methylotrophic bacteria are underlined. * *fba* sequences for *Methylomicrobium alcaliphilum* were obtained from the partially completed genome.

RuMP cycle—*Mm. alcaliphilum* and *M. flagellatus*. The FBA of these obligate methylotrophs can not be involved in the Calvin cycle due to the absence of the genes/enzymes of phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase. Although these bacteria assimilate C_1 compounds via the RuMP cycle where C_6-phosphosugars are synthesized as first products, in these methanotrophs, as in *Mc. capsulatus* Bath, hexose phosphates can be cleaved into C_3-intermediates by the enzymes of the Entner–Doudoroff pathway, i.e. bypassing the EMP pathway. In *M. flagellatus* due to the absence of the gene encoding the glycolytic enzyme phosphofructokinase (PFK), the FBA operates in the gluconeogenic direction only [23]. As for *Mm. alcaliphilum*, this methanotroph possesses PP_i-dependent PFK, which, due to reversibility of the reaction, can participate in both glycolysis and gluconeogenesis. In addition, in the *Mm. alcaliphilum* chromosome the second putative *fba* gene occurs whose translated amino acid sequence shares only 19% identity with the *Mc. capsulatus* Bath FBA and clusters with the *E. coli* FBA belonging to the type A of class II FBAs. Therefore, the additional FBA most likely operates as the glycolytic enzyme. However, properties of two aldolases of this methanotroph are not known.

The presence of CbbA-like enzymes in *Mc. capsulatus*, *Mm. alcaliphilum*, and *M. flagellatus* might imply the resemblance of the RuMP and ribulose bisphosphate (RuBP) cycles with regard to the rearrangement reactions involved in regeneration of ribulose-5-phosphate or ribulose-1,5-bisphosphate, the acceptors for C_1-compounds. Common origin of the RuMP and RuBP pathways of formaldehyde and CO_2 fixation were proposed more than 30 years ago [24]. This hypothesis might be confirmed by comparison of FBA properties, roles, and the first amino acid sequences.

The striking metabolic peculiarity of *Mc. capsulatus* Bath is the presence of the PP_i-PFK catalyzing reversible phosphorylation of both fructose-6-phosphate and sedoheptulose-7-phosphate, with higher activity and affinity to sedoheptulose-7-phosphate. This suggests that PP_i-PFK of the methanotroph belongs to the Calvin cycle enzymes [10]. Importantly, autotrophic bacteria possessing the FBAs that are most closely related to the *Mc. capsulatus* Bath FBA also have highly homologous PP_i-PFKs [10]. Thus, an evolutionary tandem of these enzymes, PP_i-PFK and FBA, functioning in the Calvin cycle, at least in some autotrophic and methylotrophic proteobacteria, can be proposed.

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