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Properties of Recombinant ATP-Dependent Fructokinase from the Halotolerant Methanotroph *Methylomicrobium alcaliphilum* 20Z

S. Y. But, O. N. Rozova, V. N. Khmelenina, A. S. Reshetnikov, and Y. A. Trotsenko*

Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, 142290 Pushchino, Moscow Region, Russia; fax: (495) 956-3370; E-mail: trotsenko@ibpm.pushchino.ru

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Abstract—In the cluster of genes for sucrose biosynthesis and cleavage in *Methylomicrobium alcaliphilum* 20Z, a gene whose encoded sequence showed high similarity to sugar kinases of the ribokinase family was found. By heterologous expression of this gene in *Escherichia coli* cells and following metal chelate affinity chromatography, the electrophoretically homogenous recombinant enzyme with six histidine residues on the C-end was obtained. The enzyme catalyzes ATP-dependent phosphorylation of fructose into fructose-6-phosphate but is not active with other sugars as phosphoryl acceptors. The fructokinase of *M. alcaliphilum* 20Z is most active in the presence of Mn^{2+} at pH 9.0 and 60°C, being inhibited by ADP (K_i = 2.50 ± 0.03 mM). The apparent K_m values for fructose and ATP are 0.26 and 1.3 mM, respectively; the maximal activity is 141 U/mg protein. The enzyme shows the highest similarity of translated amino acid sequence with putative fructokinases of methylotrophic and autotrophic proteobacteria whose *fruK* gene is located in the gene cluster of sucrose biosynthesis. The involvement of fructokinase in sucrose metabolism in *M. alcaliphilum* 20Z and other methanotrophs and autotrophs is discussed.

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Key words: fructokinases, sucrose metabolism, methylotrophic bacteria, *Methylomicrobium alcaliphilum*, *fruK*

Phosphorylation of monosaccharides is a highly important stage of carbohydrate metabolism in prokaryotes. Before assimilation and involvement in metabolism, fructose is phosphorylated either by specific enzymes of the fructose phosphoenolpyruvate-phosphotransferase system into fructose-1-phosphate or by a nucleotide-dependent fructokinase (ATP:β-D-fructose-6-phosphotransferase, EC 2.7.1.4), which catalyzes the transfer of γ-phosphate from ATP to an intracellular fructose, giving fructose-6-phosphate [1, 2]. In most heterotrophic bacteria, the genes encoding fructokinases are located on the chromosome either separately (*fruK*) [3, 4] or as a component of a sucrose utilization gene cluster (*scrK*, *sacK*, or *cscK*) that also includes genes for sugar transporters [5-7].

The aerobic halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z uses methane or methanol as the carbon and energy source but is not able to grow on sugars or other multicarbon compounds [8]. Earlier we have shown that this organism synthesizes sucrose and accumulates it in cells as an osmoprotector and/or carbon storage compound [9]. Analysis of the genome of *M. alcaliphilum* 20Z (http://www.genoscope.cns.fr/externe/English/corps_anglais.html) revealed a cluster of genes coding putative sucrose biosynthesis enzymes: sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP). In the proximity of the *sps* and *spp* genes are the gene encoding putative amylosucrase and an open reading frame (ORF) whose product shows similarity with sugar kinases belonging to the ribokinase enzymes family also denoted as the PfkB family sugar kinases. This family includes ATP-dependent enzymes: ribokinase, ketohexokinase, fructokinase, 2-dehydro-3-deoxyglucokinase, 1-phosphofructokinase, tagatose kinase, archaeal 6-phosphofructokinase, and minor 6-phosphofructokinase (PfkB) of *Escherichia coli* [10, 11]. Location of the ORF in the gene cluster of sucrose metabolism implied the possible involvement of the putative enzyme

Abbreviations: FruK, fructokinase; LB, Luria–Bertani medium; RuMP, ribulose monophosphate.

* To whom correspondence should be addressed.

in re-utilization of fructose, which is internally released from sucrose cleavage by sucrose amylase.

This work was aimed at biochemical characterization and phylogenetic analysis of the fructokinase from the halotolerant methanotroph *M. alcaliphilum* 20Z.

MATERIALS AND METHODS

Bacteria and growth conditions. *Methylomicrobium alcaliphilum* 20Z was grown under methane–air atmosphere in 2P mineral medium with the addition of 0.1 M $NaHCO_3$ and 0.3 M NaCl at 30°C [9]. *Escherichia coli* Rosetta (DE3) was grown in selective LB broth or agar (1.5% Difco agar) at 37°C [12]. Ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) were added if required.

Cloning, expression, and purification of fructokinase. The chromosomal DNA from *M. alcaliphilum* was prepared as previously described [13]. The *fruK* gene (MEALZv4_0616) was amplified using primers for PCR: forward (5′-CTCCATATGAATAAAAATCCGATCACTA-3′) and reverse (5′-TCCTCGAGGTCTAGATCCCAAGCCGCT-3′) containing recognition sites for endonucleases of restriction *Nde*I and *Xho*I, respectively. The PCR-product was purified on a Wizard column (Promega, USA), incubated with the endonucleases *Nde*I and *Xho*I, and ligated in the expression vector pET22b$^+$ preliminarily treated by these endonucleases. The resulting plasmid pET22b:*fruK* was transformed into *E. coli* Rosetta (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 25 μg/ml chloramphenicol, and cultivated until A_{600} = 0.6-0.7. The protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at the final concentration of 0.5 mM. After incubation for 12 h at 17°C, the cells were centrifuged (6000*g* for 20 min, 4°C) and resuspended in 20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 5 mM imidazole. Cell lysate was centrifuged (10,000*g*, 20 min at 4°C), and supernatant was applied to 0.5 ml Ni^{2+}-NTA column (Qiagen, Germany). After intensive washing with 20 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl and 60 mM imidazole, FruK-His_6-tag was eluted by the same buffer with 200 mM imidazole. The proteins in the fractions were analyzed by SDS-PAGE [14]. The fractions containing fructokinase were combined and stored in 40% glycerol at −20°C.

Determination of fructokinase molecular mass. The molecular mass of the enzyme was determined by gel filtration on a calibrated column with Ultrogel AcA 34 (Pharmacia, Sweden) equilibrated by 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl. Alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), chymotrypsin (25 kDa), and RNase (13 kDa) from Sigma (USA) were used as molecular weight markers.

Fructokinase activity assay. Activity of the fructokinase was measured at 30°C by using the coupling enzymes — phosphoglucose isomerase (PGI) and glucose-6-phosphate dehydrogenase (G6PDH). The assay mixture (1 ml) contained 50 mM Tris-HCl (pH 9.0), 5 mM ATP, 7.5 mM $MgCl_2$, 5 mM fructose, 0.5 mM $NADP^+$, 0.25 U of PGI, and 2 U of G6PDH. The reaction was started by addition of fructose. The reduction of $NADP^+$ was monitored on a Shimadzu UV-1700 spectrophotometer (Japan) at 340 nm.

The effect of pH on fructokinase activity was tested using the following buffers (50 mM): MES-KOH (pH 6.0-7.0), potassium phosphate (pH 6.5-5.5), Tris-HCl (pH 8.0-9.0), and sodium carbonate (pH 9.0-10.5). To determine the fructokinase thermal stability, aliquots of the enzyme were incubated in Eppendorf tubes at 30, 40, 50, and 60°C from 5 min to 1 h and rapidly cooled on ice, and the residual enzyme activity was determined at 30°C.

Kinetic parameters were determined by measuring the activity with different concentrations of fructose and ATP. The reaction mixture for measurement of the apparent K_m for fructose contained 50 mM Tris-HCl (pH 9.0), 0.5 mM $NADP^+$, 0.25 U PGI, 2 U G6PDH, 10 mM ATP, 15 mM $MgCl_2$, 0.1-20 mM fructose. Apparent K_m for ATP was measured at saturating concentration of fructose (5 mM). $MgCl_2$ concentration was either 5 mM (at ATP concentration from 0.1 to 4 mM), or 1.5-fold excess of $MgCl_2$ in relation to ATP (at ATP concentration from 5 to 20 mM) was used. The Enzyme Kinetics Module of the SigmaPlot 11 software was used for calculation of the maximal activity (A_{max}) and K_m.

When testing fructokinase substrate specificity, the enzyme activity was measured with different substrates using pyruvate kinase and lactate dehydrogenase as the coupling enzymes. The rate of NADH oxidation at 340 nm, 30°C, was evaluated in the assay mixture (1 ml) containing 50 mM Tris-HCl (pH 9.0), 4 mM ATP, 5 mM $MgCl_2$, 0.5 mM NADH, 5 mM phosphoenolpyruvate, 1 U of pyruvate kinase, and 1 U of lactate dehydrogenase. Substrates of D-glucose, D-mannose, sucrose, maltose, L-arabinose, D-xylose, fructose-6-phosphate, glucose-6-phosphate, and glucose-1-phosphate (Sigma) at concentrations 5 mM were used. Protein concentration was measured by a modified Lowry method [15].

DNA manipulations. Plasmid isolation, restriction, agarose gel electrophoresis, ligation, and transformation of *E. coli* cells were performed according to standard protocols [16]. Restriction enzymes, T4 DNA-ligase, *Taq* DNA-polymerase, and dNTP mixture were manufactured by Fermentas (Lithuania).

Sequence analyses. Sequences from the NCBI protein database (http://www.ncbi.nlm.nih.gov) were obtained by BLAST. Sequence of the *M. alcaliphilum fruK* gene was obtained from partially annotated genome (http://www.genoscope.cns.fr/externe/English/corps_

anglais.html). Sequence alignments were generated with the CLUSTAL X software (version 1.8) [17]. Minor corrections in the alignment were made manually. A dendrogram was constructed using the MEGA 4 program [18].

RESULTS

Cloning of the *fruK* gene and purification of Frk. The gene *MEALZv4_0616* coding a putative fructokinase was isolated by PCR from the genomic DNA of *M. alcaliphilum* 20Z. The amplicon between *Nde*I and *Xho*I sites was cloned into the bacterial vector pET22b(+) designed for expression of C-terminal His_6-tagged fusion proteins under control of the T7 promoter. *Escherichia coli* Rosetta (DE3) cells were transformed with the recombinant plasmid, and protein expression was induced by IPTG addition in the early-log phase of culture. Electrophoretically homogenous preparation of the recombinant protein was obtained by metal affinity chromatography.

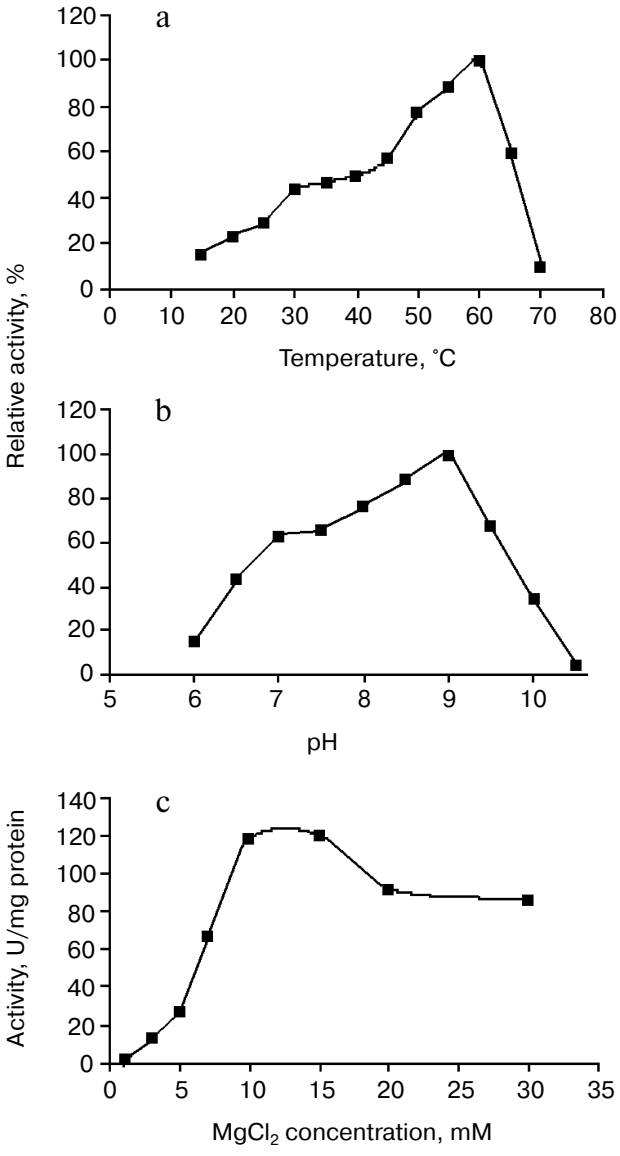

Fig. 1. Effect of temperature (a), pH (b), and concentrations of Mg^{2+} ions at 10 mM ATP (c) on fructokinase activity.

The molecular mass ~35 kDa of the protein determined by SDS-PAGE was in good agreement with that calculated from predicted amino acid sequence (34.5 kDa). According to gel filtration on the column with Ultrogel AcA 34, the molecular mass of the recombinant enzyme corresponded to its monomeric form (data not shown).

The recombinant protein catalyzed ATP-dependent phosphorylation of fructose into fructose-6-phosphate but was not active with D-glucose, D-mannose, D-xylose, L-arabinose, maltose, sucrose, fructose-6-phosphate, glucose-6-phosphate, or glucose-1-phosphate as phosphoryl acceptors. The following compounds were used as phosphate donors (relative activity): ATP (100%), GTP (52.2%), and UTP (21.5%). Activity was not detected with CTP and PPi. The enzyme activity was dependent on divalent cations (relative activity): Mg^{2+} (100%), Mn^{2+} (146%), Co^{2+} (63%), Zn^{2+} (1.1%), and Ni^{2+} (0.8%). The enzyme was inactive without a metal cofactor.

The highest enzyme activity was registered at 60°C (Fig. 1a). The enzyme was stable for at least two months at 4°C in 0.2 mM imidazole buffer (pH 8.0). The enzyme activity slightly decreased after 1 h incubation at 30°C. After 1 h incubation at 40°C, the residual enzyme activity was about 80%, and after 5 min incubation at 50°C – 20%. The enzyme activity was completely lost after 30 min at 60°C.

The fructokinase was active in the pH range 6.5-9.0 being maximally active at pH 9.0, and sharply decreased at more alkaline pH values (Fig. 1b).

Kinetics. The initial reaction rate was assayed at different concentrations of fructose and ATP. The enzyme exhibited maximal activity at total ATP and Mg^{2+} concentration ratio from 1 : 1 to 1 : 1.5 (Fig. 1c). At the ratio of Mg^{2+}/ATP less than 1, fructokinase activity sharply decreased. It is possible that the enzyme uses MgATP complex as a phosphate group donor and is inhibited by free ATP or requires high Mg^{2+} concentration [2].

The K_m for fructose was measured at saturated ATP concentration (10 mM) and at excessive but not inhibiting concentration of Mg^{2+} (15 mM). The enzyme exhibited a typical Michaelis–Menten kinetics. The apparent K_m for fructose was 0.26 ± 0.03 mM. The apparent K_m for ATP assayed at saturated fructose concentration (5 mM) was 1.3 ± 0.2 mM. A_{max} was 141 ± 6 U/mg protein. ADP inhibited the enzyme activity (K_i = 2.5 mM at 5 mM fructose, 7.5 mM $MgCl_2$, and 4 mM ATP). The enzyme activity decreased by 50% with the addition of 400 mM NaCl in the reaction mixture.

Phylogeny of fructokinases. Blast analysis of bacterial genomes presented in database revealed genes homologous

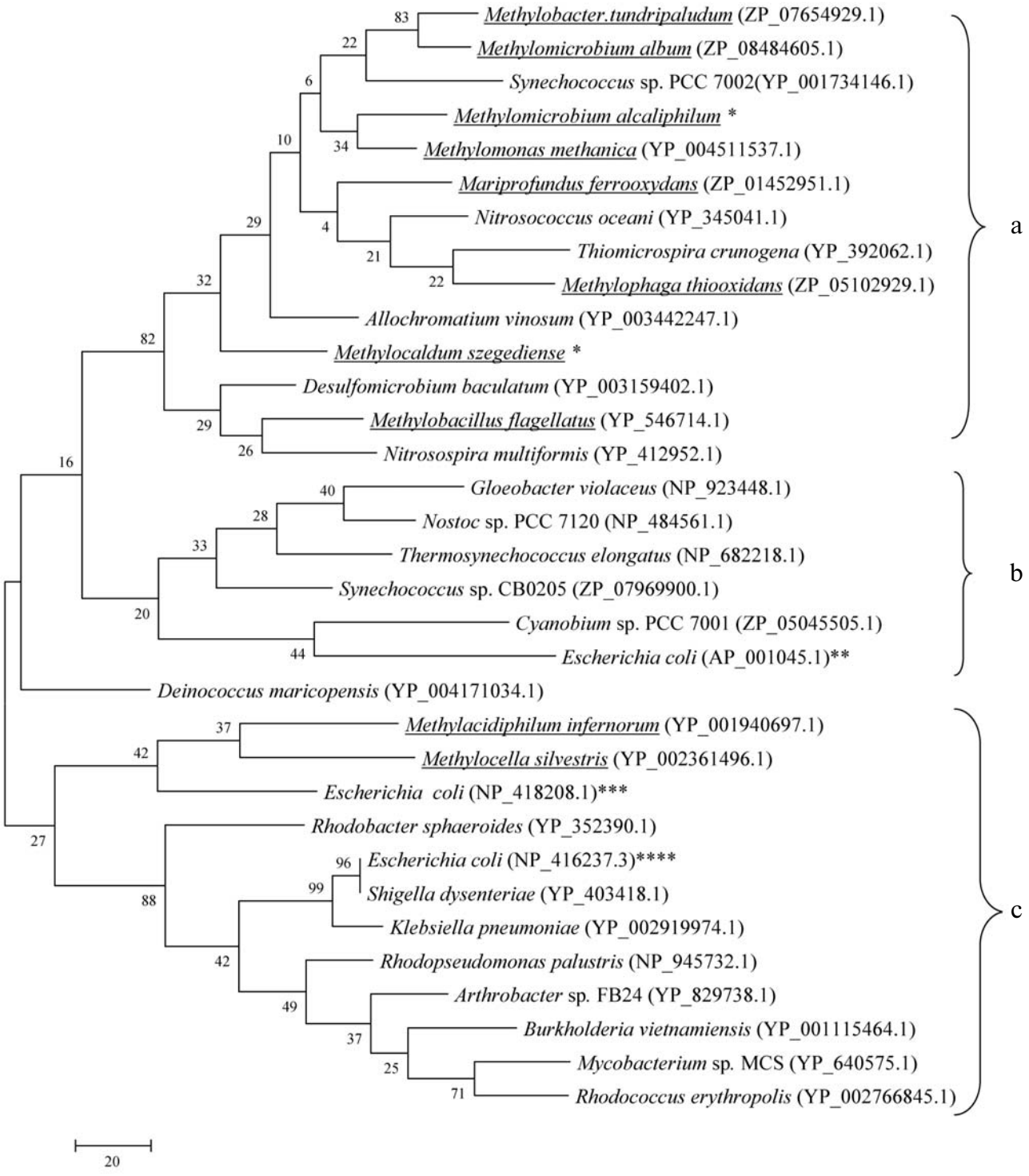

Fig. 2. Phylogenetic tree of some sugar kinases. Methylotrophic bacteria are underlined. a) Genes encoding fructokinases are located in the proximity of the sucrose biosynthesis genes; b) genes encoding fructokinases are located separately on the chromosome; c) genes of sugar kinases, annotated as PfkB-family kinases. * Sequence of gene *fruK* from *M. szegediense* O-12 was obtained from the partially finished genome; ** fructokinase of *E. coli*; *** ribokinase of *E. coli*; **** the minor 6-phosphofructokinase (PfkB) of *E. coli*.

to *fruK* of *M. alcaliphilum* 20Z in other methanotrophs: *Methylomonas methanica* MC09 (61.4% identity of translated amino acid sequences), *Methylobacter tundripaludum* SV96 (54.4%), and *Methylomicrobium album* BG8 (54.9%). Other close relatives of the *M. alcaliphilum* 20Z fructokinase are those from cyanobacteria and autotrophic proteobacteria, as well as from methylobacteria not growing on methane *Methylophaga thiooxydans* (47.6%) and *Methylobacillus flagellatus* (40.3%) (Fig. 2). In the bacteria listed, the *fruK* genes were located adjacently to sucrose biosynthesis genes *sps* and *spp*. A homolog of fructokinase is present in the thermophilic methanotroph *Methylocaldum szegediense* (41.1% identity). The fructokinases of these bacteria comprised a separate branch on the phylogenetic tree of sugar kinases (Fig. 2).

DISCUSSION

In this work, the biochemical properties of fructokinase from the halotolerant methanotroph *M. alcaliphilum* 20Z were first determined. The peculiarities of this enzyme are monomeric organization as well as obligate specificity and high affinity to fructose. The high pH optimum corresponded to alkaliphilic character of the methanotroph optimally growing at pH 9.0. The relatively high K_m for ATP (1.3 mM) and inhibition by ADP might imply its optimal functioning at conditions of high energy status of the cells. Contrarily, the low value of the apparent K_m for fructose (0.26 mM) possibly implies the capacity of the strain to metabolize this compound at its low intracellular concentrations.

The strain 20Z does not grow on fructose or other sugars; therefore, the role of fructokinase in the methanotroph is not associated with assimilation of the exogenous sugars. Using Blast analysis, genes homologous to *fruK M. alcaliphilum* 20Z were revealed in the published genomes of other obligate methanotrophs: *M. methanica* MC09, *M. tundripaludum* SV96, *M. album* BG8, *M. szegediense* O-12, and some autotrophic proteobacteria and cyanobacteria, as well as in the methylotrophs not growing on methane *M. thiooxidans* and *M. flagellatus* (Fig. 2). In these bacteria, the *fruK*-like genes, annotated as *pfkB* genes, are adjacent to the gene cluster coding sucrose biosynthesis enzymes – sucrose phosphate synthase and sucrose phosphate phosphatase.

Importantly, the listed methanotrophic bacteria and some autotrophs (*Mariprofundus ferrooxydans*, *Synechococcus* sp. PCC 7002, *Desulfomicrobium baculatum*, *Thiomicrospira crunogena*) possess the 4-gene cluster *sps–spp–fruK–as*, containing, besides sucrose biosynthesis genes and fructokinase gene, the gene encoding amylosucrase, which cleaves sucrose into glucose and fructose and is capable of transferring glucosyl residue from sucrose to glycogen. Other members of this branch including methylobacteria not utilizing CH_4, possess the

Properties of fructokinases from some microorganisms

Parameter	*Methylomicrobium alcaliphilum* 20Z (this work)	*Thermococcus litoralis* [21]	*Bifidobacterium longum* [22]	*Zymomonas mobilis* [3]	*Lactococcus lactis* [5]
Molecular mass, kDa (number of subunits)	35 (1)	70 (2)	35	56 (2)	64 (2)
pH-optimum of activity	9.0	7.5-8.0	6.0	7.4	7.0
T_{opt}, °C	60	80	50	–	–
Substrate specificity	fructose	fructose	fructose	fructose, mannose	fructose, mannose
K_m, mM					
fructose	0.26	2.3	0.74	0.7	0.31
ATP	1.3	0.81	0.77	0.45	0.59
A_{max}, U/mg protein	141	920	1.8	350	–
Relative activity with phosphoryl donors	ATP (100%) > GTP (52%) > UTP (22%) > CTP (0%), PP_i (0%)	ATP (100%) > ITP (73%) > GTP (62%) > UTP (16%), CTP (16%)	ATP (100%) > ITP (78%) > GTP (23%) > CTP (6%) > TTP (0.18%)	–	ATP > GTP > ITP > UTP

three-gene cluster *sps–spp–fruK*. Another exception is the thermotolerant methanotroph *M. szegediense* O-12 possessing *fruK* outside the sucrose biosynthesis gene cluster since it is separated by divergently located genes encoding proteins with unknown function. This methanotroph synthesizes sucrose as a thermoprotectant in response to increasing growth temperature [19]. The fructokinases of methylotrophic bacteria and some autotrophic proteobacteria together comprised a separate branch on the phylogenetic tree of sugar kinases (Fig. 2).

With the exception of *M. alcaliphilum* 20Z, the methylotrophs listed are not halotolerant and sucrose accumulation in their cells has not been studied. Obviously, sucrose biosynthesis in methylotrophic and autotrophic bacteria may be related not so much to osmoadaptation, but to other metabolic features. The presence of fructokinase activating endogenous fructose, which is formed as a result of sucrose cleavage implies the possible role of the disaccharide as a carbon reserve compound. In other methanotrophs – the thermoacidiphilic verrucomicrobial *Methylacidiphilum infernorum* and acidotolerant alphaproteobacterium *Methylocella silvestris* – the genes of sugar kinases annotated as the *pfkB* genes, have low homology with *fruK* of *M. alcaliphilum* 20Z (22.1 and 16.2% identity, respectively), and are not the components of the sucrose biosynthesis gene cluster.

Thus, in this work the fructokinase involved in reutilization of endogenous forming sucrose is first characterized. Earlier, fructokinases were studied in heterotrophic microorganisms where the respective genes are a part of cluster for exogenous sucrose utilization, which also includes the genes for sugar transporters, while the genes of sucrose biosynthesis are absent: *Zymomonas mobilis* [4], *Lactococcus lactis* [5], *E. coli* [11, 20] (table).

In conclusion, the strict substrate specificity of fructokinases of *M. alcaliphilum* 20Z to fructose allows the use this enzyme in clinical and analytical biochemistry as a specific reagent for fructose determination including mixtures containing other carbohydrates. Also, fructokinase from *M. alcaliphilum* 20Z is relatively stable, active in a wide range of pH and at high ionic strength, thus additionally favoring the application of the enzyme for bioanalytical purposes.

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