Characterization of Fructose 1,6-Bisphosphatase and Sedoheptulose 1,7-Bisphosphatase from the Facultative Ribulose Monophosphate Cycle Methylotroph *Bacillus methanolicus*

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The genome of the facultative ribulose monophosphate (RuMP) cycle methylotroph *Bacillus methanolicus* encodes two bisphosphatases (GlpX), one on the chromosome (GlpX^C) and one on plasmid pBM19 (GlpX^P), which is required for methylotrophy. Both enzymes were purified from recombinant *Escherichia coli* and were shown to be active as fructose 1,6-bisphosphatases (FBPases). The FBPase-negative *Corynebacterium glutamicum* Δfbp mutant could be phenotypically complemented with *glpX^C* and *glpX^P* from *B. methanolicus*. GlpX^P and GlpX^C share similar functional properties, as they were found here to be active as homotetramers *in vitro*, activated by Mn²⁺ ions and inhibited by Li⁺, but differed in terms of the kinetic parameters. GlpX^C showed a much higher catalytic efficiency and a lower K_m for fructose 1,6-bisphosphate ($86.3 \text{ s}^{-1} \text{ mM}^{-1}$ and $14 \pm 0.5 \text{ }\mu\text{M}$, respectively) than GlpX^P ($8.8 \text{ s}^{-1} \text{ mM}^{-1}$ and $440 \pm 7.6 \text{ }\mu\text{M}$, respectively), indicating that GlpX^C is the major FBPase of *B. methanolicus*. Both enzymes were tested for activity as sedoheptulose 1,7-bisphosphatase (SBPase), since a SBPase variant of the ribulose monophosphate cycle has been proposed for *B. methanolicus*. The substrate for the SBPase reaction, sedoheptulose 1,7-bisphosphate, could be synthesized *in vitro* by using both fructose 1,6-bisphosphate aldolase proteins from *B. methanolicus*. Evidence for activity as an SBPase could be obtained for GlpX^P but not for GlpX^C. Based on these *in vitro* data, GlpX^P is a promiscuous SBPase/FBPase and might function in the RuMP cycle of *B. methanolicus*.

Bacillus methanolicus is a Gram-positive, thermotolerant, and facultative methylotrophic bacterium (1–3) that can use the one-carbon (C₁) compound methanol as a source of carbon and energy. A variety of different enzymes and pathways for C₁ metabolism have been described among methylotrophs (4, 5). In *B. methanolicus*, methanol utilization is initiated by its oxidation to formaldehyde catalyzed by methanol dehydrogenase (Mdh) (3), and it was recently shown that this bacterium has three genes, all encoding active Mdhs (6). The generation of reduction equivalents occurs via oxidation to CO₂ catalyzed by formaldehyde dehydrogenase and formate dehydrogenase (7, 8).

Formaldehyde fixation in the ribulose monophosphate (RuMP) pathway is initiated by the fixation of formaldehyde to ribulose 5-phosphate (Ru5-P) by hexulose 6-phosphate synthase (Hps), followed by conversion to fructose 6-phosphate (F6-P) by phosphohexuloisomerase (Phi) (Fig. 1). Regeneration of Ru5-P involves enzymes shared with glycolysis and the pentose phosphate pathway (9) (Fig. 1). F6-P is phosphorylated by phosphofructokinase (PFK). Fructose 1,6-bisphosphate (FBP) is cleaved to glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) by fructose 1,6-bisphosphate aldolase (FBA). *B. methanolicus* possesses a chromosomally encoded FBA (FBA^P) and a plasmid-encoded FBA (FBA^C) (10). FBA^P is the major gluconeogenic FBA since it shows a >10-fold-higher catalytic efficiency for aldol condensation than FBA^C. FBA^C is the major glycolytic FBA in this bacterium, since it shows a >30-fold-higher catalytic efficiency for FBP cleavage than FBA^P (10).

Two different variants of the regeneration part of the RuMP pathway are known for the conversion of triosephosphates and F6-P to Ru5-P: the TA (transaldolase) variant and the SBPase (sedoheptulose 1,7-bisphosphatase) variant. Three enzymes, transketolase (TKT), ribose 5-phosphate isomerase (RPI), and

ribulose 5-phosphate 3-epimerase (RPE), are shared in both variants. In the TA variant, erythrose 4-phosphate (E4-P) and F6-P are directly converted to GAP and sedoheptulose 7-phosphate (S7-P) catalyzed by TA, and FBA functions as in glycolysis, i.e., catalyzing the cleavage of FBP to GAP and DHAP.

In the SBPase variant, S7-P is generated in two reactions. First, E4-P and DHAP are condensed to sedoheptulose 1,7-bisphosphate (SBP) by sedoheptulose 1,7-bisphosphate aldolase (SBA) (possibly FBA^P or FBA^C), and subsequently, SBP is dephosphorylated to S7-P by a SBPase (possibly GlpX encoded on plasmid pBM19 [GlpX^P] or GlpX encoded on the chromosome [GlpX^C]). The reactions of SBA and SBPase are characteristic of the regeneration part of the Calvin cycle in photosynthetic organisms (11), and overproduction of SBPase in tobacco was shown to enhance carbon assimilation and crop yield (12). Recently, *Saccharomyces cerevisiae* was shown to possess a promiscuous SBPase encoded by *SHB17*, which also has FBPase and octulose-bisphosphatase (OBPase) activity and operates in the riboneogenesis pathway (13). It is possible that a promiscuous bisphosphate aldolase is active as both FBA and SBA and also that a bisphosphatase active as FBPase and SBPase exists. Alternatively, separate enzymes catalyze the individual reactions. Based on its genome sequence, *B.*

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FIG 1 Proposed map of the biochemical reactions of the methanol oxidation and assimilation pathways in *B. methanolicus*, including the TA (dashed arrows) and the SBPase (solid arrows) variants of the RuMP cycle. MDH, methanol dehydrogenase (EC 1.1.1.244); HPS, 3-hexulose-6-phosphate synthase (EC 4.1.2.43); PHI, 6-phospho-3-hexuloisomerase (EC 5.3.1.27); PFK, 6-phosphofructokinase, (EC 2.7.1.11); FBA, fructose-bisphosphate aldolase (EC 4.1.2.13); TKT, transketolase (EC 2.2.1.1); GlpX, fructose-bisphosphatase (EC 3.1.3.1); TA, transaldolase (EC 2.2.1.2); RPE, ribulose-phosphate 3-epimerase (EC 5.1.3.1); RPI, ribose-5-phosphate isomerase (EC 5.3.1.6); H6-P, 3-hexulose 6-phosphate; F6-P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; E4-P, erythrose 4-phosphate; SBP, sedoheptulose 1,7-bisphosphate; S7-P, sedoheptulose-7-phosphate; R5-P, ribose 5-phosphate; X5P, xylose 5-phosphate; Ru5P, ribulose 5-phosphate. The reactions are described in detail in the text.

methanolicus possesses the whole genetic equipment for both variants of the RuMP cycle (14, 15). Except for TA and RPI, all enzymes of the RuMP cycle regeneration phase are encoded by two alternative genes in *B. methanolicus*, either on the naturally occurring plasmid pBM19 or on the chromosome.

It is not clear why *B. methanolicus* harbors two distinct sets of genes for the regeneration part of the RuMP cycle. However, it has been shown that curing of the natural plasmid pBM19, which carries the key *mdh* gene and five genes with deduced roles in the RuMP cycle (*glpX*, *fba*, *tkt*, *pfk*, and *rpe*), resulted in the loss of the ability to grow on methanol and caused higher methanol tolerance and reduced formaldehyde tolerance (15). Transcription of *mdh*, all five plasmid-borne RuMP cycle genes, as well as the chromosomal genes *hps* and *phi* was increased during growth with methanol, suggesting their importance for methylotrophy (16). While pBM19 is critical for growth on methanol and is important for formaldehyde detoxification, the maintenance of this plasmid represents a burden for *B. methanolicus* when growing on mannitol. Methanol consumption by this organism involves the concerted recruitment of both plasmid and chromosomal genes, and this discovery represented the first documentation of plasmid-dependent methylotrophy (14, 15, 17).

This work focused on the biochemical characterization of the aldolases GlpX^P and GlpX^C from *B. methanolicus*. FBPase (EC 3.1.3.11) hydrolyzes FBP to inorganic phosphate and F6-P. FBPases are members of the large superfamily of Li⁺-sensitive phosphatases. This group is divided into the inositol phosphatases and the FBPases. Generally, these enzymes are characterized by their requirement for divalent metal ions and their Li⁺ sensitivity

(18). Based on their amino acid sequences, five different classes of FBPases (FBPases I to V) have been identified (19–22). FBPase I, the most widely distributed FBPases, is found in most prokaryotes, a few archaea, and all eukaryotes (19, 21, 23, 24). FBPase II is present in *Escherichia coli*, encoded by *glpX*, and in *Synechocystis* sp. strain PCC6803 (25). FBPase III is present, e.g., in *Bacillus subtilis* (encoded by *fbp*) (26), and FBPase IV is present in *Pyrococcus furiosus* (encoded by *fbpA*) (27), *Methanococcus jannaschii* (28), and *Archaeoglobus fulgidus* (29). FBPases of class V are represented by the FBPases TK2164 from *Pyrococcus* (*Thermococcus*) *kodakaraensis* and ST0318 from *Sulfolobus tokodaii* (20, 30). Recently, class V FBPase in the (hyper)thermophilic archaea *Ignicoccus hospitalis*, *Metallosphaera sedula*, and *Thermoproteus neutrophilus* was described as a promiscuous enzyme (FBP aldolase/phosphatase) (31, 32). Eukaryotes possess only the FBPase I enzyme. Class I, II, and III FBPases are found primarily in bacteria, class IV is found primarily in archaea, and class V is found primarily in thermophiles (21, 23). Some microorganisms possess more than one FBPase, mostly combinations of class I and II FBPases, as in *E. coli* (19), or class II and III FBPases, as found in *B. subtilis* (26, 33).

FBPases show a very close functional and structural relationship to SBPases (EC 3.1.3.37) (34). Recent phylogenetic studies showed that SBPases and FBPases share an evolutionary origin (35). SBPases catalyze the reversible dephosphorylation of SBP to S7-P. In the Calvin cycle, both SBPase and FBPase operate. While in photosynthetic bacteria, such as cyanobacteria, a single promiscuous enzyme carries out both reactions (36), in green plants, two separate enzymes catalyze the individual reactions. SBPases are homodimeric, comprising two identical subunits of 35 to 38 kDa, and are immunologically distinct from FBPase (37, 38).

Here, we provide evidence that GlpX^C catalyzes hydrolysis of FBP with a high catalytic efficiency ($86.3 \text{ s}^{-1} \text{ mM}^{-1}$) and that GlpX^P is a promiscuous enzyme active both as a SBPase and as a FBPase albeit with a low catalytic efficiency ($8.8 \text{ s}^{-1} \text{ mM}^{-1}$). Moreover, experimental evidence for the synthesis of SBP by both aldolases (FBA^P and FBA^C) from *B. methanolicus* was obtained. Based on these *in vitro* results, the SBPase variant of the RuMP cycle may operate *in vivo* during methylotrophic growth of *B. methanolicus*.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strain DH5 α was used as a standard cloning host (39). Recombinant cells were grown in lysogeny broth (LB) medium at 37°C supplemented with ampicillin (100 μ g/ml), chloramphenicol (15 μ g/ml), kanamycin (50 μ g/ml), spectinomycin (100 μ g/ml), and 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) when appropriate. Recombinant *E. coli* procedures were performed as described previously (40). Recombinant protein production was carried out with *E. coli* BL21(DE3) as the host (41).

The *Corynebacterium glutamicum* wild-type (WT) strain (ATCC 13032) and the derived Δfbp mutant (42) lacking FBPAse were used for the heterologous expression of *glpX* genes from WT *B. methanolicus* MGA3 (ATCC 53907). Plasmid pEKEx3 was used for IPTG-inducible expression of *glpX^C* (GI 40074240) and *glpX^P* (GI 2716575) (43). *C. glutamicum* strains were cultured in LB medium or CgXII minimal medium (44). For growth experiments, *C. glutamicum* cells were harvested from cultures grown in LB medium overnight by centrifugation ($3,220 \times g$ for 10 min), washed in CgXII medium, and used to inoculate CgXII minimal medium. All growth experiments with *C. glutamicum* were carried out in baffled shake flasks at 30°C and 120 rpm. Growth was monitored by

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Function and/or relevant characteristic(s) ^a	Reference or source
Strains		
<i>B. methanolicus</i> strain MGA3	WT strain	1
<i>E. coli</i>		
DH5α	General cloning host [<i>F</i> [−] <i>thi-1 endA1 hsdR17</i> (r [−] m [−]) <i>supE44 ΔlacU169</i> (80 <i>lacZ</i> ΔM15) <i>recA1 gyrA96 relA1</i>]	Bethesda Research Laboratories
BL21(DE3)	Host for recombinant protein production [<i>F</i> [−] <i>ompT hsdSB</i> (r _B [−] m _B [−]) <i>gal dcm</i> (DE3)]	Novagen
<i>C. glutamicum</i>		
ATCC 13032	WT strain; auxotrophic for biotin	65
Δ <i>fbp</i> mutant	In-frame deletion of the <i>fbp</i> gene of the WT	42
Plasmids		
pEKEx3	Spec ^r ; <i>C. glutamicum</i> - <i>E. coli</i> shuttle vector (<i>P</i> _{lac} <i>lacI</i> ^q pBL1 <i>oriV_{Cg}</i> <i>oriV_{Ec}</i>)	43
pEKEx3- <i>glpX</i> ^C (Bme)	Derived from pEKEx3, for regulated expression of <i>glpX</i> ^C (GI 40074240) of <i>B. methanolicus</i>	This work
pEKEx3- <i>glpX</i> ^P (Bme)	Derived from pEKEx3, for regulated expression of <i>glpX</i> ^P (GI 2716575) of <i>B. methanolicus</i>	This work
pEKEx3- <i>fbp</i> (Cgl)	Derived from pEKEx3, for regulated expression of <i>fbp</i> (cg1019) of <i>C. glutamicum</i>	This work
pHP13	<i>B. methanolicus</i> - <i>E. coli</i> shuttle vector; Clm ^r	66
pTH1	Similar to pHP13 but with a <i>mdh</i> promoter upstream of the MCS	This work
pTH1- <i>glpX</i> ^C (Bme)	Derived from pTH1, for regulated expression of <i>glpX</i> ^C of <i>B. methanolicus</i>	This work
pTH1- <i>glpX</i> ^P (Bme)	Derived from pTH1, for regulated expression of <i>glpX</i> ^P of <i>B. methanolicus</i>	This work
pET16b	Amp ^r ; T7lac; vector for His-tagged protein overproduction	Novagen
pET16b- <i>fbp</i> ^C (Bme)	Purification of His-tagged <i>B. methanolicus</i> FBA ^C from <i>E. coli</i> BL21(DE3)	This work
pET16b- <i>fbp</i> ^P (Bme)	Purification of His-tagged <i>B. methanolicus</i> FBA ^P from <i>E. coli</i> BL21(DE3)	This work
pET16b- <i>glpX</i> ^C (Bme)	Purification of His-tagged <i>B. methanolicus</i> GlpX ^C from <i>E. coli</i> BL21(DE3)	This work
pET16b- <i>glpX</i> ^P (Bme)	Purification of His-tagged <i>B. methanolicus</i> GlpX ^P from <i>E. coli</i> BL21(DE3)	This work

^a Abbreviations: Spec^r, spectinomycin resistance; Clm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; MCS, multiple cloning site.

determination of the optical density at 600 nm (OD₆₀₀) until the stationary phase.

B. methanolicus strains were grown at 50°C in the following media. SOBsuc medium is SOB medium (Difco) supplemented with 0.25 M sucrose. Solid medium was described previously (45). Mannitol growth of *B. methanolicus* was performed with Mann₁₀ medium containing salt buffer, 1 mM MgSO₄, vitamins, trace metals, 0.025% yeast extract (Difco), and mannitol (10 g/liter) (pH 7.2). Mann_{10-Y} medium is Mann₁₀ medium without yeast extract (pH 7.0). Methanol growth of *B. methanolicus* was performed in MeOH₂₀₀ medium, which is similar to Mann₁₀ medium except that the mannitol is replaced with methanol (200 mM). Bacterial growth was performed in shake flasks (500 ml) in 50 ml medium at 200 rpm and monitored by measuring the OD₆₀₀. The inoculation of the precultures for all growth experiments with *B. methanolicus* strains was performed with frozen ampoules of *B. methanolicus* as a starter culture. Ampoules of *B. methanolicus* cells were prepared from exponentially growing cultures (OD₆₀₀ of 1.0 to 1.5) and stored at −80°C in 15% (vol/vol) glycerol (14). For inoculation, ampoules were thawed, and a 250-μl cell suspension was used to inoculate 50 ml Mann₁₀ or MeOH₂₀₀ medium. A total of 5 to 10% of these cultures, when grown to an OD₆₀₀ of 5 to 7, was used to inoculate fresh and prewarmed media for growth experiments.

DNA manipulation. Plasmids and genomic DNA from *B. methanolicus* were isolated by using Qiagen Midiprep and DNeasy tissue kits (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. The transformation of plasmid pTH1 and its derivatives into *B. methanolicus* MGA3 was performed by using electroporation, as described previously (14, 45).

Homologous overexpression of *fbp* in *C. glutamicum*. For overexpression of *fbp* (cg1019), the gene was amplified by PCR using genomic DNA of WT *C. glutamicum* and oligonucleotide primers *fbp*-Cgl-fw and *fbp*-Cgl-rv (primer sequences are listed in Table 2). The resulting PCR product of *fbp* was ligated into SmaI-restricted, IPTG-inducible vector pEKEx3, resulting in pEKEx3-*fbp*(Cgl). Sequencing confirmed the integrity of the insert.

Heterologous expression of *glpX*^C and *glpX*^P from *B. methanolicus* in *C. glutamicum*. PCR products from one chromosomal gene (*glpX*^C [GI 415883782]) and one plasmid-borne gene (*glpX*^P [GI 2716575]) were generated from genomic DNA as well as plasmid pBM19 DNA from *B. methanolicus* MGA3 by PCR using oligonucleotide primer pair *glpX*_P-Bme-fw and *glpX*_P-Bme-rv and oligonucleotide primer pair *glpX*_C-Bme-fw and

TABLE 2 Sequences of oligonucleotides used

Oligonucleotide	Sequence (5'–3') ^a
<i>fbp</i> -Cgl-fw	GGATCCGAAAGGAGGCCCTTCAGATGCCTATC GCAACTCCCG
<i>fbp</i> -Cgl-rv	GGATCCTTACTTAGAGGTGGTCTTTCCAAC
<i>glpX</i> _P -Bme-fw	TTTTCATGTGCCA TTAGTTTCAATGAAG
<i>glpX</i> _P -Bme-rv	TTTGAATTCCTTAAGCTTTACCTGAAGATCCA
<i>glpX</i> _C -Bme-fw	TTTTCATGTGCCCTTAGTTTCAATGACGGAA
<i>glpX</i> _C -Bme-rv	TTTTGGTACCTTACGCTTTTCCGGAAGAACCG
<i>glpX</i> _P -Bme-w	TTTTCATGTGCCATTAGTTTCAATGAAG
<i>glpX</i> _P -Bme-rv	TTTTGAGCTCTTAAGCTTTACCTGAAGATCCA
<i>glpX</i> _C -Bme-fw	CTCGGATCCGAAAGGAGGCCCTTCAGATGCCA TTAGTTTCAATGAAGG
<i>glpX</i> _C -Bme-rv	CTCGAGCTCGCGTTAAGCTTTACCTGAAGATCC
<i>glpX</i> _C -Bme-fw	GGCGCATATGCCCTTAGTTTCAATGAC
<i>glpX</i> _C -Bme-rv	GGCGCATATGTTACGCTTTTCCGGAAGAAC
<i>glpX</i> _P -Bme-fw	GGCGCATATGCCATTAGTTTCAATGAAGAT
<i>glpX</i> _P -Bme-rv	GCGGCATATGTTAAGCTTTACCTGAAGATC
<i>fbp</i> _P -Bme-fw	GCGGCATATGAGGGAATTGAAAAGCGAAAA
<i>fbp</i> _P -Bme-rv	GCGGCATATGTTATGATAAGCTTCAATAAATTG GTATT
<i>fbp</i> _C -Bme-fw	GCGACTCGAGATGGAAGAAGTTTAAACAAT
<i>fbp</i> _C -Bme-rv	GCGTCTCGAGTTAAGTTTGATCACTAAGT

^a Restriction sites are in boldface type, linker sequences for crossover PCR and ribosomal binding sites are shown in italics, and stop and start codons are underlined.

glpX_C-Bme-fw (Table 2). The amplified product of *B. methanolicus* was restricted by BamHI and SacI, and the resulting PCR product was ligated into BamHI- and SacI-restricted vector pEKEx3. The resulting vectors were named pEKEx3-*glpX^C*(Bme) and pEKEx3-*glpX^P*(Bme). Vector pEKEx3 allows IPTG-inducible gene expression in *C. glutamicum* and *E. coli*. All resulting vector inserts were sequenced to confirm their sequence integrity.

Homologous overexpression of the two *glpX* genes in *B. methanolicus*. Overexpression vector pTH1 was used to allow methanol-inducible expression of *B. methanolicus glpX* genes. This vector is analogous to plasmid pHP13, in which the strong *mdh* promoter was cloned in frame with the *mdh* ribosome binding site (RBS) region to allow methanol-inducible expression in *B. methanolicus* (15, 46). The 1-kb DNA fragments of the *glpX^C* and *glpX^P* coding regions were amplified from DNA of *B. methanolicus* by using primer pair *glpX_P*-Bme-fw and *glpX_P*-Bme-rv and primer pair *glpX_C*-Bme-fw and *glpX_C*-Bme-rv (Table 2). The resulting PCR products were digested with PciI and ligated into the PciI-digested vector pTH1, yielding vectors pTH1-*glpX^C*(Bme) and pTH1-*glpX^P*(Bme), respectively.

Protein purification. For protein production with *E. coli* BL21(DE3) (41), *glpX^P* and *glpX^C* were amplified by PCR using primer pair *glpX_C*-Bme-fw and *glpX_C*-Bme-rv and primer pair *glpX_P*-Bme-fw and *glpX_P*-Bme-rv (Table 2). The resulting PCR products were ligated, after restriction with NdeI or XhoI, into NdeI- and XhoI-restricted pET16b (Novagen, Madison, WI, USA), resulting in pET16b-*glpX^C* and pET16b-*glpX^P*. The pET16b vector allows the production of an N-terminal decahistidine-tagged FBA in *E. coli* BL21(DE3). Protein production and purification were performed as described previously (47). Both enzymes were purified to homogeneity. After purification, the His tag was cleaved by factor Xa (Novagen, San Diego, CA) according to the manufacturer's recommendations and buffered in 20 mM Tricine (pH 7.7). Protein purification was analyzed by 12% SDS-PAGE (48). The protein concentration was measured by using a Bradford protein assay (Bio-Rad) with bovine serum albumin (BSA) as the standard.

Molecular mass determination of GlpX proteins. The quaternary structures of the GlpX proteins were determined by gel filtration, as described previously (47), using 1 mg GlpX dissolved in 2 ml of 20 mM Tricine (pH 7.7).

Preparation and measurements of GlpX activity in crude extracts of *B. methanolicus*. Crude cell extracts were prepared based on a protocol described previously (15). *B. methanolicus* cells harboring plasmids pTH1, pTH1-*glpX^C*, and pTH1-*glpX^P* were grown in SOB medium with 0.25 mM sucrose to stationary phase (OD_{600} , 2.5 to 3.3). Gene expression was induced by the addition of 200 mM methanol at the very beginning of the experiment. Twenty milliliters of the cell culture was harvested by centrifugation (4,000 rpm for 10 min at 4°C), washed in 50 mM potassium phosphate buffer (pH 7.5), and stored at -20°C. The cells were disrupted by sonication, as previously described (17). Cell debris was removed by centrifugation (14,000 × g for 1 h at 4°C), and the supernatant was collected as crude extracts. Protein concentrations were determined by a Bradford assay (Bio-Rad), using bovine serum albumin as a standard. FBPase activity was measured according to standard conditions (FBP cleavage toward F6-P).

Enzyme assays for the purified GlpX proteins *in vitro*. Determination of the FBA activity in the direction of FBP cleavage toward F6-P was done by a NADPH-linked enzyme assay with the coupling enzyme phosphoglucose isomerase (PGI) (from *Saccharomyces cerevisiae*; Sigma), glucose-6-phosphate dehydrogenase (G6PDH) (from *Leuconostoc mesenteroides*; Sigma), and recombinant GlpX from *B. methanolicus* (42). The standard reaction mixture (final volume, 1 ml) contained 20 mM Tricine buffer (pH 7.7), 0.25 mM NADP, 2 mM Mn_2Cl_2 , 100 mM KCl, 0.4 U/ml G6PDH, 0.7 U/ml PGI, and purified GlpX protein, which was preheated for 4 min at 50°C. NADPH oxidation ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) was monitored at 340 nm on a Shimadzu UV1700 spectrophotometer. The reaction was initiated by the addition of FBP (the final concentration

varied from 0.05 to 10 mM). The pH optimum was defined by using the following buffers (50 mM) under standard conditions: acetate (pH 5.0 to 6.0), phosphate (pH 6.0 to 7.0), Tris-HCl (pH 7.0 to 9.0), and glycine-NaOH (pH 9.0 to 10.0). The pH was adjusted at 50°C. The effect of metal ions and EDTA on phosphatase activity was measured under standard conditions in the presence of Zn^{2+} , Ca^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Mg^{2+} , Fe^{2+} , Mn^{2+} , Ni^{2+} , and K^+ at a 1 mM final concentration in the reaction mixture. The remaining percent activities were determined by comparison with a mixture with no metal ion added. To investigate the effect of EDTA, an EDTA salt solution was incubated with FBP for 4 min. The measurement was done according to standard assay procedures with a 1 mM EDTA final concentration in a 1-ml reaction mixture. To study the thermal stability of the GlpX proteins, the assay mixture described above was prepared in 1.5-ml reaction tubes and incubated for up to 2 h at 30°C to 70°C. Samples were taken periodically, and the residual enzyme activity was measured under standard conditions in a separate reaction mixture.

The substrate specificities of both GlpX^C and GlpX^P were determined by the quantification of inorganic phosphate that is formed by hydrolysis of potential substrates. Therefore, EnzCheck phosphate determination reagents (Molecular Probes, Eugene, OR, USA) were used in a coupled assay according to the manufacturer's instructions. The released phosphate and 2-amino-6-mercapto-7-methyl-purine riboside (MESG) were converted by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methyl-purine in a solution containing 20 mM Tricine (pH 7.7), 2 mM Mn_2Cl_2 , 100 mM KCl, 0.2 mM 2-amino-6-mercapto-7-methyl-purine riboside, purine nucleoside phosphorylase (1 U/ml), purified His-tagged GlpX protein (2 mg/ml), and 1 mM the substrate to be tested. The formation of 2-amino-6-mercapto-7-methyl-purine was monitored at 360 nm.

Determination of the FBA activity in the direction of SBP synthesis was done by using a discontinuous, coupled enzyme assay mixture containing TKT (from *S. cerevisiae*; Sigma), recombinant GlpX^C and GlpX^P, as well as FBA^C and FBA^P from *B. methanolicus*. Because E4-P is not acquired by purchase, E4-P was generated in a prereaction by using the TKT (5 U/mg) from F6-P and GAP. Protein production and purification were done as previously described (47). The purified protein was buffered in 50 mM Tris-HCl (pH 7.5). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 20 mM F6-P, 20 mM GAP, 20 mM DHAP, 10 μ M thiamine pyrophosphate (TPP), 2 mM $MnCl_2$, and 3 U/mg of each purified enzyme (FBA^C or FBA^P and GlpX^C or GlpX^P). The reaction was started by the addition of TKT (5 U/mg) to the mixture. The detection of the generated products was performed via liquid chromatography-mass spectrometry (LC-MS) as described below. The assay was performed at 50°C for 45 min in a volume of 1 ml. The reaction was stopped by purification of the mixture containing enzymes by using an Amicon Ultra-0.5 centrifugal filter (Millipore) according to the manufacturer's specifications.

LC-MS analysis of the products after enzyme assays. LC-MS data were obtained by using a LaChromUltra (Hitachi Europe, United Kingdom) high-performance liquid chromatography (HPLC) system coupled to a microTOF-Q hybrid quadrupole/time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany). For ionization, the mass spectrometer was equipped with an electrospray ionization (ESI) source. Separation of the samples via HPLC was carried out with a SeQuant ZIC-PHILLIC column (150 by 2.1 mm; Merck KGaA, Darmstadt, Germany), using 10 mM ammonium bicarbonate solution (pH 9.3) as eluent A and acetonitrile as eluent B. The injection volume was 2 μ l, the flow rate was set to 150 μ l min⁻¹, and gradient elution was performed as follows: 80% eluent B at a time (*t*) of 0 min, 10% eluent B at a *t* of 30 min, 10% eluent B at a *t* of 35 min, 80% eluent B at a *t* of 40 min, and 80% eluent B at a *t* of 60 min. MS detection was performed via the ESI source in negative-ionization mode. Nitrogen was applied as sheath, dry, and collision gas. For internal mass calibration, a solution of formate (0.1 M) in 50% (vol/vol) isopropanol was injected for each MS analysis. Tandem MS (MS/MS) analyses were performed by using the auto-MS/MS mode of the microTOF-Q instrument (see Table S1 in the supplemental material).

FIG 2 Primary sequence alignment of the *B. methanolicus* GlpX proteins with FBpase II homologs. Black boxes indicate identical residues in all 6 organisms, and gray boxes indicate highly conserved residues. Abbreviations: *B. methanolicus* (C), *B. methanolicus* MGA3 FBpase encoded on the chromosome (NCBI accession number ZP_11545811); *B. methanolicus* (P), *B. methanolicus* FBpase encoded by pBM19 (accession number ZP_11548894); *Synechocystis*, *Synechocystis* sp. PCC6803 FBpase (accession number NP_441738); *E. coli*, *E. coli* FBpase GlpX (accession number P0A9C9); *M. tuberculosis*, *M. tuberculosis* H37Rv FBpase (accession number NP_215615); *C. glutamicum*, *C. glutamicum* FBpase Fbp (accession number NP_600242). Bars above the sequences indicate highly conserved domains of the Li⁺ binding site. The sequence alignment was carried out by using CLUSTALW, and the alignment was formatted by using BoxShade.

Raw data were analyzed by using Compass software 1.3 (Bruker Daltonics, Bremen, Germany). Automatic internal mass calibration was achieved by using the high-performance computing (HPC) quadratic algorithm. Identification of compounds was performed either by determining the specific mass-to-charge ratio and the retention time or by comparing the fragment ions in the MS/MS mode (see Table S2 in the supplemental material).

Computational analysis. Sequence comparisons were carried out with protein sequences obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/>), and the sequence alignment of the *B. methanolicus* MGA3 GlpX proteins and other class II FBpases was done by using CLUSTALW (49) and formatted with BoxShade.

RESULTS

Bioinformatic analysis and phylogeny of the FBpases GlpX^P and GlpX^C from *B. methanolicus*. *B. methanolicus* MGA3 possesses two genes encoding putative FBpases, *glpX^C* and *glpX^P*, putatively encoding proteins of 321 amino acids and 320 amino acids, respectively. The deduced primary sequences of these proteins show a similarity of 72% (226/310 amino acids) and an identity of 54% (167/310) to each other. The closest homolog of GlpX^C present in the database is the chromosomally encoded protein reported under NCBI accession number ZP_10121059.1 (98% identical amino acids), from *B. methanolicus*

strain PB1. Similarly, the closest homolog of plasmid-encoded GlpX^P is the protein reported under accession number ZP_10132906.1, from *B. methanolicus* strain PB1 (97% identical amino acids), which is encoded on plasmid pBM20. BLAST analyses of the amino acid sequences of GlpX^C and GlpX^P as queries suggested their classification as type II FBpases, with >100 sequences of class II FBpases sharing 50% or more identical amino acids. Primary sequence alignment with biochemically characterized class II FBpases, including *Synechocystis* sp. PCC6803 Fbp1 (25), *E. coli* GlpX (16), *Mycobacterium tuberculosis* H37Rv Rv1099c (50), and *C. glutamicum* Fbp (42), revealed >50 conserved amino acid residues (Fig. 2). Four blocks of conserved residues are highlighted in black in Fig. 2 (VIG EGE, APML, AVDP, and DGDV). The third block is part of the Li⁺-sensitive phosphate motif and was shown previously by crystallographic and mutagenesis studies to be important but not sufficient for metal ion binding and catalysis (51). While FBpase II enzymes from *E. coli* (52), *M. tuberculosis* (50), *Synechocystis* sp. PCC6803 (25), *B. subtilis* (33), and *C. glutamicum* (42) have been biochemically characterized, the characteristics of a promiscuous FBpase/SBpase from a nonphotosynthetic bacterium lacking the Calvin cycle have not yet been determined.

TABLE 3 Growth rates and FB Pase activities of various *C. glutamicum* strains^a

<i>C. glutamicum</i> strain	Growth on 100 mM fructose		Growth on 100 mM acetate	
	Mean μ (h) $\pm$ SD	Mean FB Pase activity (U/mg) $\pm$ SD	Mean μ (h) $\pm$ SD	Mean FB Pase activity (U/mg) $\pm$ SD
WT(pEKEEx3)	0.36 $\pm$ 0.042	0.022 $\pm$ 0.003	0.28 $\pm$ 0.095	0.024 $\pm$ 0.081
Δfbp (pEKEEx3)	NG	ND	NG	ND
Δfbp [pEKEEx3- <i>fbp</i> (Cgl)]	0.32 $\pm$ 0.019	0.024 $\pm$ 0.005	0.27 $\pm$ 0.011	0.023 $\pm$ 0.008
Δfbp [pEKEEx3- <i>glpX</i> ^C (Bme)]	0.35 $\pm$ 0.052	0.022 $\pm$ 0.005	0.26 $\pm$ 0.002	0.025 $\pm$ 0.008
Δfbp [pEKEEx3- <i>glpX</i> ^P (Bme)]	0.29 $\pm$ 0.031	0.021 $\pm$ 0.009	0.21 $\pm$ 0.021	0.024 $\pm$ 0.005

^a *C. glutamicum* was grown in CgXII medium containing 100 mM fructose or 100 mM acetate. Data represent mean values and standard deviations of three independent replicates. μ , growth rate; NG, no growth; ND, not determined.

Overexpression of *glpX*^C and *glpX*^P results in increased FB Pase activity in *B. methanolicus* crude extracts. In order to experimentally confirm that the *glpX*^C and *glpX*^P genes encode active FB Pases, both genes were overexpressed in *B. methanolicus*. Plasmids pTH1-*glpX*^C and pTH1-*glpX*^P, which allow methanol-inducible overexpression, were constructed and used to transform *B. methanolicus*. FB Pase activities were determined in crude extracts of the transformants after growth in complex medium with and without methanol present as an inducer. As expected, *B. methanolicus* MGA3 and MGA3 carrying the empty vector pTH1 showed comparable FB Pase activities regardless of whether methanol was present as an inducer or not (0.077 $\pm$ 0.003 U/mg under noninducing conditions and 0.081 $\pm$ 0.009 U/mg with methanol). While the overexpression strains carrying either pTH1-*glpX*^C or pTH1-*glpX*^P showed FB Pase activities of 0.090 $\pm$ 0.004 and 0.093 $\pm$ 0.003 U/mg, respectively, in the absence of methanol, induction by methanol resulted in significantly (2- to 3-fold) increased FB Pase activities of 0.243 $\pm$ 0.092 and 0.187 $\pm$ 0.064 U/mg, respectively. Thus, overexpression of *glpX*^C and *glpX*^P indeed increased FB Pase activities, indicating that both genes encode functionally active FB Pases.

Both *glpX*^C and *glpX*^P from *B. methanolicus* complement growth deficiencies of the FB Pase-deficient *C. glutamicum* Δfbp mutant strain. To test if the two FB Pases from *B. methanolicus* can function in glycolysis and/or gluconeogenesis *in vivo*, the FB Pase-deficient *C. glutamicum* Δfbp mutant (42) was used as a host for genetic complementation experiments (Table 3), since gene-directed deletion mutagenesis is not possible in *B. methanolicus*. The *C. glutamicum* Δfbp strain is known to be unable to grow on acetate or other gluconeogenic substrates, such as citrate, glutamate, or lactate, as the sole source of carbon and lacks detectable FB Pase activity in crude extracts (42). FB Pase is also essential for growth of *C. glutamicum* on fructose as the sole carbon source. Fructose is taken up into *C. glutamicum* cells by the phosphotransferase system (PTS) and is concomitantly phosphorylated to fructose 1-phosphate (not to the glycolytic intermediate F6-P). Fructose 1-phosphate is then phosphorylated to FBP. Thus, for provision of F6-P and subsequently of glucose 6-phosphate and, thus, for growth on fructose, hydrolysis of FBP by FB Pase is required. To complement the *C. glutamicum* Δfbp strain, *fbp* from *C. glutamicum* as well as *glpX*^P and *glpX*^C from *B. methanolicus* were cloned into the IPTG-inducible expression vector pEKEEx3, and the resulting vectors were used to transform the *C. glutamicum* Δfbp strain. Expression of both *glpX*^P and *glpX*^C from *B. methanolicus*, as well as the endogenous *C. glutamicum* gene, led to FB Pase activities similar to those of WT *C. glutamicum* and restored the

ability of the *C. glutamicum* Δfbp strain to grow with fructose or with the gluconeogenic carbon source acetate (Table 3). As expected, growth of the *C. glutamicum* Δfbp strain with glucose was not affected and was comparable in all strains tested (data not shown). Thus, heterologous expression of both FB Pase-encoding genes from *B. methanolicus* led to sufficient FB Pase activities to support growth of a *C. glutamicum* Δfbp strain on a gluconeogenic carbon source such as acetate.

Recombinant production, purification, and biochemical characterization of GlpX^P and GlpX^C. Both *glpX*^P and *glpX*^C were PCR amplified and cloned into pET16b for production of the enzymes with an N-terminal His tag (Table 1). The resulting plasmids were transformed into *E. coli* BL21(DE3), and protein production was induced by the addition of IPTG to exponentially growing cells. After Ni-nitrilotriacetic acid (NTA) chromatography, His tags were cleaved by using factor Xa, and the enzymes were buffered in 20 mM Tricine (pH 7.7). Protein purifications from 500 ml of culture broth led to average concentrations of 1 mg/ml for both enzymes and a total amount of about 4 mg protein per purification.

The optimal assay conditions of the enzymes as FB Pases were determined by using a coupled spectrometric assay for measuring the formation of F6-P from FBP (as described in Materials and Methods). Measurements were performed in 20 mM Tricine buffer at 50°C and with substrate concentrations of 0.2 mM for GlpX^C and 2 mM for GlpX^P, which is at least about 5-fold higher than the determined K_m values. Activity could be measured for both enzymes within a broad pH range of between 7 and 10 and an optimum pH range of between 8.5 and 9.0. All subsequent assays were performed at pH 7.7, the putative physiologically relevant pH of *B. methanolicus*. The enzymes were also purified and stored at pH 7.7 and were found to be stable (data not shown). Gel filtration of both proteins and FB Pase activity assays showed that both proteins eluted in a single fraction, indicating that they are active as homotetramers, with molecular masses for the tetramers of about 135 kDa for GlpX^C and about 142 kDa for GlpX^P.

The presence of divalent metal cations was required for activity for both GlpX proteins. Different metal ions were tested at final concentrations of 1 mM, but only Mn²⁺ supported the activity of both proteins. Other divalent metal ions, including Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Fe²⁺, and Ca²⁺, showed no significant activation of FB Pase at the tested concentrations. Replacement of Mn²⁺ with Mg²⁺ resulted in an almost complete loss of activity for GlpX^C and GlpX^P. The addition of EDTA at an equimolar concentration to the bivalent metal ions strongly reduced FB Pase activity. KCl increased the activity of GlpX^C by 20% at a concentration of 100 mM

but had no effect on GlpX^P. A residual activity of about 50% was observed for both FBPs of *B. methanolicus* when 1 mM the monovalent cation Li⁺ was present.

To identify inhibitors or activators of FBPs activity, potential effectors were tested at concentrations of 1 mM. GlpX^P and GlpX^C were both inhibited by ATP (50% and 56%, respectively) and ADP (33% and 38%, respectively), whereas AMP had no effect on the two enzymes. All other tested effectors, such as PEP, F1-P, F6-P, and fructose 2,6-bisphosphate, showed no significant effect on both FBPs at concentrations of up to 5 mM. Only GlpX^C showed inhibition by higher FBP concentrations (K_i value of 3.5 mM [see below]).

Temperature optima and stability of the purified GlpX^C and GlpX^P proteins. To test the temperature profile of the two GlpX proteins from *B. methanolicus*, activity was measured under standard conditions in a dehydrogenase/isomerase-coupled assay under conditions without limitation by the coupling enzymes. Under the chosen conditions, both GlpX proteins displayed the highest activity at a temperature of around 55°C, which is similar to the optimal growth temperature of *B. methanolicus*. Temperatures higher than this resulted in strongly decreased FBPs activities, which could be, to some extent, explained by the instability of the substrates triose phosphate and fructose bisphosphate (32). Thermal stability was tested from 30°C to 70°C by incubating the enzyme for different periods in 20 mM Tricine (pH 7.7) and 2 mM Mn²⁺ prior to determining the activity at 50°C. Both GlpX proteins from *B. methanolicus* remained stable at 30°C, 40°C, and 50°C for at least 2 h. Temperatures of 60°C and higher led to a complete loss of activity within 20 min for GlpX^C and within 10 min for GlpX^P (see Fig. S1 and S2 in the supplemental material).

Kinetic parameters and substrate spectrum of the FBPs from *B. methanolicus*. The kinetic parameters of GlpX^C and GlpX^P for hydrolysis of FBP were determined at 50°C and at pH 7.7 in 20 mM Tricine with 2 mM MnCl₂ (and 100 mM KCl for GlpX^C). The activity of both GlpX proteins followed Michaelis-Menten kinetics for the substrate FBP (data not shown). Only for GlpX^C was substrate inhibition observed, with a K_i value of 3.5 mM. The K_m for chromosomally encoded GlpX^C was calculated to be 14 ± 0.5 μM FBP, and the activity was maximal at 2 ± 0.11 U/mg (Table 4). On the other hand, the plasmid-encoded enzyme GlpX^P exhibited an ~30-fold-higher K_m of 440 ± 7.6 μM and an ~3-fold-higher V_{max} of 7 ± 0.32 U/mg than GlpX^C. The purified GlpX proteins displayed catalytic efficiencies (k_{cat}/K_m) as FBPs of 86.3 s⁻¹ mM⁻¹ for GlpX^C and 8.8 s⁻¹ mM⁻¹ for GlpX^P (Table 4). To determine the substrate specificity of *B. methanolicus* FBPs, the rate of enzyme-catalyzed formation of inorganic phosphate from various potential substrates was measured. Neither GlpX^P nor GlpX^C accepted the structurally related F1-P, F6-P, glucose 6-phosphate, mannose 6-phosphate, and glycerol phosphate as substrates (all present at 1 mM). SBP could not be tested directly, since it is not commercially available. The ~50-fold-higher catalytic efficiency of GlpX^C than of GlpX^P indicated that GlpX^C is the major FBPs of *B. methanolicus*.

Development of a novel assay for synthesis and hydrolysis of SBP *in vitro* by combinations of purified FBA proteins and FBPs from *B. methanolicus* MGA3. In the SBPase variant of the RuMP cycle, SBP is produced from E4-P and DHAP by SBA and dephosphorylated to yield S7-P by SBPase. Unfortunately, since neither E4-P nor SBP is commercially available, these compounds cannot be used directly in enzyme assays to obtain

TABLE 4 Biochemical properties of GlpX^P and GlpX^C

Parameter	Value	
	GlpX ^P	GlpX ^C
Molecular masses (kDa)	33, 132 (tetramer)	35, 145 (tetramer)
Optimal conditions	20 mM Tricine (pH 7.7), 2 mM Mn ²⁺ , 50°C	20 mM Tricine (pH 7.7), 7 mM Mn ²⁺ , 100 mM KCl, 50°C
Optimal pH	8.5–9	8.5–9
Optimal temp (°C)	55	55–60
Temp stability (°C)	<60	≤50
Kinetics		
FBPase ^a		
Mean K_m (μM) ± SD	440 ± 7.6	14 ± 0.5
Mean V_{max} (U/mg) ± SD	7 ± 0.32	2 ± 0.11
k_{cat} (s ⁻¹)	3.9	1.2
k_{cat}/K_m (s ⁻¹ mM ⁻¹)	8.8	86.3
SBPase ^b	+	–

^a Values for K_m , V_{max} , and catalytic efficiency (k_{cat}/K_m) were determined for two independent protein purifications, and mean values and arithmetic deviations from the mean are given.

^b –, SBP cannot be utilized as a substrate; +, SBP serves as a substrate.

evidence for synthesis and hydrolysis of SBP. To circumvent this limitation, a coupled discontinuous enzyme assay including transketolase from *S. cerevisiae* was used. E4-P and xylulose 5-phosphate (XU5-P) were generated from F-6P and GAP by transketolase from *S. cerevisiae*. Aldol condensation of E-4P with DHAP to yield SBP was tested for by using purified FBA^C or FBA^P from *B. methanolicus* (10). Subsequently, hydrolysis of SBP to S7-P was assayed by using purified GlpX^C or GlpX^P from *B. methanolicus*. The reactions were carried out for 30 min at 50°C, and substrates, intermediates, and products were identified and quantified by LC-MS using available standards. The identities of the sugar bisphosphates FBP and SBP were verified via MS/MS analysis (see Materials and Methods for details). Various combinations of substrates and enzymes were tested (Fig. 3). No evidence for instabilities of the sugar phosphates at 50°C was obtained when the substrates were incubated without enzymes.

Incubation of F6-P and GAP with TKT from *S. cerevisiae* led to the formation of E4-P and Xu5-P. When DHAP and either FBA^C or FBA^P from *B. methanolicus* were present in addition, formation of SBP could be detected. Since standards were not available, and due to possible ion suppression effects of ESI-MS detection, only estimates of the relative concentrations of FBP, S7-P, and SBP could be derived. When TKT from *S. cerevisiae* and FBA^C or FBA^P from *B. methanolicus* were present, the ratio between SBP and FBP was between 1:1.3 and 1:1.5 (data not shown). LC-MS/MS analysis confirmed the identity of SBP, which showed the expected mass shift of 30 Da (–CHOH–) compared to FBP (Fig. 3C). Thus, these results indicated that both FBAs from *B. methanolicus* are active as SBAs *in vitro*.

Hydrolysis of SBP and formation of S7-P occurred only when GlpX^P, but not GlpX^C, was added (Fig. 3). When GlpX^P was present in addition to TKT from *S. cerevisiae* and FBA^C or FBA^P from *B. methanolicus*, the ratio of SBP, S7-P, and FBP was 1:0.2:0.9 (estimates of the relative concentrations of S7-P, SBP, and FBP [data not shown]). Thus, for the major FBPs of *B. methanolicus*, GlpX^C, evidence for SBPase activity could not be obtained *in vitro*, while GlpX^P showed activity as an SBPase.

Taken together, the observed synthesis of SBP by either FBA^C

FIG 3 Determination of the sugar phosphate intermediates of the RuMP cycle using liquid chromatography-mass spectrometry (LC-MS). The sample was analyzed after performing a discontinuous enzyme assay for 30 min at 50°C. Details are given in Materials and Methods. The peaks for the given sugar phosphates were identified by using characteristic mass spectra. For the identification of sedoheptulose 1,7-bisphosphate and fructose 1,6-bisphosphate, MS/MS was used. (A) Scheme of the substrate and enzyme combinations used in the assay. X indicates presence in the assay. (B) Presence (check marks) or absence of the indicated sugar phosphates, as detected by LC-MS/MS analysis. (C) LC-MS spectra of FBP and SBP. Abbreviations: FBA, fructose-bisphosphate aldolase (EC 4.1.2.13); TKT, transketolase (EC 2.2.1.1); GlpX, fructose-bisphosphatase (EC 3.1.3.1); F6-P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; SBP, sedoheptulose 1,7-bisphosphate; S7-P, sedoheptulose-7-phosphate; X5P, xylulose 5-phosphate.

or FBA^P and the hydrolysis of SBP to S7-P by GlpX^C demonstrate that the SBPase variant of the RuMP cycle is operative *in vitro* and corroborates the hypothesis that it may be active *in vivo* during methylotrophic growth of *B. methanolicus*.

DISCUSSION

B. methanolicus has the complete set of genes for the methanol assimilatory pathway, of which *hps*, *phi*, *rpi*, and *tal* are solely found on the chromosome, while additional copies of *mdh* and some RuMP cycle genes can be found on the naturally occurring plasmid pBM19. Based on the gene annotations, two variants of the RuMP cycle appear possible. This work deals with the question of whether the chromosomal and plasmid-encoded bisphosphatases GlpX^C and GlpX^P, key enzymes of the regeneration part of the RuMP cycle, are active as FBPases and/or SBPases.

Based on their amino acid sequences, both GlpX proteins from *B. methanolicus* belong to class II FBPases. The FBPases from *B. methanolicus* can be clearly distinguished from each other due to (i) their catalytic efficiencies as FBPases and (ii) their activities as SBPases. As FBPases, they share some biochemical properties; e.g., they both are active as homotetramers, require Mn²⁺ as divalent cations, and exhibit pH optima between 8.5 and 9. Their similar thermal stabilities and temperature optima correlate well with the physiology of thermophilic *B. methanolicus*, which is able to grow at temperatures of between 35°C and 60°C (1). It is peculiar that the optimal temperature, especially for GlpX^C, in the *in vitro* activity assay was higher than the temperature at which the enzyme is stable, but this is not unprecedented and has also been described for both FBAs of *B. methanolicus* (10) and for the FBAs of *Bacillus*

stearothermophilus (53) and *Anoxybacillus gonensis* (54). However, a diverse stability of the GlpX enzymes *in vitro* cannot be excluded. Both *glpX^C* and *glpX^P* complemented the FBPase-negative *C. glutamicum* Δfbp strain, and both FBPases were inhibited similarly by EDTA, Li⁺, ATP, and ADP. As in *E. coli* (19, 52), inhibition by ATP and ADP is thought to prevent futile cycling between phosphofructokinase and FBPase during growth on glycolytic carbon sources (55).

GlpX^C clearly is the preferred FBPase in *B. methanolicus*, as it shows a much higher catalytic efficiency with FBP as the substrate ($k_{cat}/K_m = 86.3 \text{ s}^{-1} \text{ mM}^{-1}$ for GlpX^C) than GlpX^P ($k_{cat}/K_m = 8.8 \text{ s}^{-1} \text{ mM}^{-1}$). The K_m value of $14 \pm 0.5 \text{ }\mu\text{M}$ GlpX^C is similar to those of other class II FBPases: 14 μM , 12 to 17 μM , 20 μM , and 35 μM for GlpX from *C. glutamicum*, *M. tuberculosis*, *B. subtilis*, and *E. coli*, respectively (18, 19, 33, 42). The finding that expression of *glpX^C* is not notably induced during a shift to growth in methanol (14, 56) coincides with its function as a major FBPase and sets it apart from the SBPase/FBPase encoded by *glpX^P*, which is induced when shifted to methanol.

The presence of more than one FBPase, as described here for *B. methanolicus*, is known for many bacteria, and mostly combinations of class I and II FBPases or class II and III FBPases have been found (19). *E. coli* possesses three FBPases, one class I FBPase, encoded by *fbp* (16), and two class II FBPases, encoded by *glpX* (19) and *yggF* (52). The type I FBPase, probably the main FBPase in *E. coli*, is essential for growth on gluconeogenic substrates (21, 23) and is inhibited strongly by AMP and to a lesser extent by PEP (16, 57). The *E. coli* class II FBPases GlpX and YggF have been shown to possess Mn²⁺-dependent FBPase activity with distinct

catalytic properties but are dispensable for growth with gluconeogenic substrates (19, 52). Both class II FBPases exhibit lower affinity and catalytic efficiency toward FBP than Fbp. Both class II FBPases are sensitive to Li^+ and inorganic phosphate and are inhibited by ATP and ADP, while only GlpX is stimulated by PEP (19, 52). As expression of *glpX* is induced by glycerol and glycerol 3-phosphate, GlpX is supposed to be important under these conditions rather than being active as a general FBPase under gluconeogenic conditions (58). The role of YggF in *E. coli*, which is encoded in an operon together with mannitol phosphoenolpyruvate-dependent transferase, is still unknown. *B. subtilis* has a class III FBPase (*fbp*) (26) and a class II FBPase, encoded by *ywjI* (33). The Mn^{2+} -dependent activity of Fbp is inhibited by AMP, which could be abolished by PEP, whereas for YwjI, an inhibitory effect of PEP was observed (33). In contrast to *E. coli*, it has been shown that *B. subtilis* Δfbp and $\Delta ywjI$ strains were both still able to grow on gluconeogenic substrates such as fructose, glycerol, or malate (59), whereas the double mutant was unable to grow on carbon sources demanding FBPase activity (33). Thus, both FBPases are able to bypass each other during gluconeogenesis, indicating a functional equivalence of both FBPases from *B. subtilis* (33).

Only GlpX^P from *B. methanolicus*, the less preferred FBPase (see above), was found to show SBPase activity, which is commensurate with its primary role as an SBPase. As SBP is not available commercially, demonstration of SBPase activity required a coupled discontinuous enzyme assay and subsequent LC-MS/MS analysis of the sugar (bis)phosphates. Unfortunately, this assay did not allow the determination of kinetic parameters for SBP hydrolysis. To date, promiscuous FBPases/SBPases have been found only in the Calvin cycle of proteobacteria and cyanobacteria, while in higher plants and algae, two distinct gene products specific as FBPase and SBPase exist (34, 60). Two promiscuous FBPases/SBPases were identified in *Alcaligenes eutrophus*, a facultative chemoautotroph, which also assimilates CO_2 via the Calvin cycle when growing autotrophically with hydrogen or formate as the energy source (61). Also, *Synechococcus* sp. PCC7942, an obligate autotroph, contains two isoforms of FBPases: one form participates in the Calvin cycle in chloroplasts, and the other form is involved in gluconeogenesis in the cytoplasm. Only isoform I shows a promiscuous FBPase/SBPase activity (25). The methanotroph *Methylococcus capsulatus* Bath possesses an aldolase, which is additionally active as an SBPase (62), whereas an SBPase from this bacterium has not yet been characterized. This organism is known to have 3 pathways for formaldehyde and CO_2 assimilation: the RuMP cycle, the serine pathway, and the Calvin pathway (63). Since *B. methanolicus* has no active Calvin cycle, GlpX^P is the first promiscuous FBPase/SBPase from nonphotosynthetic bacteria lacking the Calvin cycle. However, the first demonstration of a promiscuous FBPase/SBPase in a nonphotosynthetic organism was SHB17 from *S. cerevisiae*, which was shown to function in riboneogenesis (13).

In *B. methanolicus*, SBP is synthesized by both aldolase enzymes FBA^C and FBA^P, as revealed by the coupled discontinuous enzyme assay and subsequent LC-MS/MS analysis, as described above. While it was shown previously that both enzymes are active in the cleavage of FBP as well as in the reverse aldol condensation reaction leading to FBP, the catalytic efficiencies allowed distinguishing them. FBA^C is the major glycolytic enzyme and FBA^P is the major gluconeogenic one from *B. methanolicus* (10). As E4-P is no longer commercially available, kinetic parameters could not

be determined, and it remains to be shown whether one of the enzymes shows a higher catalytic efficiency for the aldol condensation of E4-P and DHAP to SBP. Taking into account that only *fbp*^P is induced during a shift to growth in methanol, FBA^P might play a more important role during methylotrophy and, thus, might be the primary SBP aldolase in *B. methanolicus*.

It is tempting to speculate that the SBPase variant of the RuMP cycle is more biologically relevant in *B. methanolicus* than the TA variant. TA is encoded by the chromosomal *tal* gene, but *tal* is not induced during a shift to growth on methanol (14, 56). Moreover, it remains to be shown whether *tal* encodes a functionally active TA. Importantly, this study provides evidence that both enzymatic reactions of the SBPase variant are active *in vitro*: FBA^P and FBA^C can synthesize SBP, and GlpX^P hydrolyzes SBP to S7-P. The genes for the key enzymes in the SBPase variant (*fbp*^P and *glpX*^P) are induced on methanol. Thus, the methanol-induced synthesis of these enzymes and their *in vitro* activities support an important role of the SBPase variant of the RuMP cycle in *B. methanolicus*.

Hitherto, it has not been possible to delete genes in *B. methanolicus*, but some loss-of-function data with respect to methylotrophy are available. Plasmid pBM19 is necessary for growth of *B. methanolicus* in methanol (15) but appears to represent a metabolic burden during growth in mannitol (14). Apparently, one or more of the chromosomal RuMP cycle genes cannot make up for the loss of their plasmid-borne copy. By serendipity, it was found that a particular isolate of MGA3 was able to grow with methanol, although FBA^P was nonfunctional due to a point mutation (10). Thus, the RuMP cycle operates with only FBA^C, GlpX^P, and TA being present.

Taken together, a more complex view of methylotrophy in *B. methanolicus* emerged, since its genome sequence has been determined (56) and since some biochemical properties of multiple copies of methanol dehydrogenase (6) and RuMP cycle enzymes (6, 14, 15, 62, 64; this study) have been elucidated. In addition, it can be anticipated that our understanding of methylotrophic growth by *B. methanolicus* will be furthered in particular by omics and carbon flux analyses.

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