

## Molecular and Biochemical Characterization of a Distinct Type of Fructose-1,6-Bisphosphatase from *Pyrococcus furiosus*

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**The *Pyrococcus furiosus* *fbpA* gene was cloned and expressed in *Escherichia coli*, and the fructose-1,6-bisphosphatase produced was subsequently purified and characterized. The dimeric enzyme showed a preference for fructose-1,6-bisphosphate, with a  $K_m$  of 0.32 mM and a  $V_{max}$  of 12.2 U/mg. The *P. furiosus* fructose-1,6-bisphosphatase was strongly inhibited by  $Li^+$  (50% inhibitory concentration, 1 mM). Based on the presence of conserved sequence motifs and the substrate specificity of the *P. furiosus* fructose-1,6-bisphosphatase, we propose that this enzyme belongs to a new family, class IV fructose-1,6-bisphosphatase.**

The hyperthermophilic archaeon *Pyrococcus furiosus* is capable of metabolizing sugar via an Embden-Meyerhof-like pathway. A combination of physiological, biochemical, and genetic studies has revealed that pyrococcal glycolysis differs from the regular Embden-Meyerhof pathway by its incorporation of new conversions, its novel enzymes, and its unique control (5). Compelling evidence of the deviation of pyrococcal glycolysis from the canonical glycolysis includes the recruitment of two unique ADP-dependent sugar kinases (10, 21) and the presence of a structurally distinct phosphoglucose isomerase (23) and a glyceraldehyde-3-phosphate ferredoxin oxidoreductase (22) in *P. furiosus*. In addition, the genes encoding the homologous and distantly related fructose-1,6-bisphosphate aldolase and phosphoglycerate mutase were recently predicted, and their functions were subsequently confirmed experimentally (19; J. van der Oost, unpublished data). The remaining glycolytic and gluconeogenic enzymes were easily identified in the genome sequence of *P. furiosus*. However, no gene coding for a homolog of the gluconeogenic fructose-1,6-bisphosphatase (EC 3.1.3.11) (FBPase) could be identified. This observation also holds true for other archaea, with the exception of *Halobacterium* sp. strain NRC1, which contains a classical FBPase (13).

FBPase is an essential regulatory enzyme in the gluconeogenic pathway. It converts D-fructose-1,6-bisphosphate to D-fructose-6-phosphate, an important precursor in biosynthetic pathways. Generally, a divalent metal ion such as  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Co^{2+}$ , or  $Zn^{2+}$  is required for catalytic activity. The three-dimensional structures of several FBPases have been elucidated (9, 24, 26), and all exhibit a typical sugar phosphatase fold (<http://scop.mrc-lmb.cam.ac.uk/scop>).

It has recently been reported that the inositol monophosphatase (I-1-Pase) (EC 3.1.3.25) from *Methanococcus jannaschii* (MJ0109) exhibits FBPase activity, and it has been suggested that this enzyme might be the missing FBPase in archaea (20). In addition, MJ0109 orthologs in *Archaeoglobus fulgidus* and *Thermotoga maritima* show FBPase activity (20).

In an attempt to complete the set of determined glycolytic and gluconeogenic enzymes in *P. furiosus*, we cloned and expressed the MJ0109 ortholog from *P. furiosus* (48% identity on the amino acid level) in *Escherichia coli* and investigated its ability to function as a thermoactive FBPase.

**Transcript analysis and cloning of *fbpA*.** An ortholog (*fbpA*) of MJ0109 (2) was identified in the *P. furiosus* genome database (<http://www.genome.utah.edu>). This ortholog was originally designated as an extragenic suppressor, *suhB*. The start of the *fbpA* gene was predicted based on the presence and proper spacing of a potential Shine-Dalgarno sequence and on multiple alignment of the deduced amino acid sequence with those of related enzymes (Fig. 1). To test whether the *fbpA* gene was transcribed in *P. furiosus*, total RNA was isolated from a pyruvate-grown *P. furiosus* culture (40 mM) as described previously (25). The presence of the *fbpA* transcript was confirmed (data not shown) by using a reverse transcription-PCR system according to the instructions of the manufacturer (Promega) with 1  $\mu$ g of *P. furiosus* RNA and the primers BG977 and BG978 (see below). Moreover, a recent genome-based microarray analysis of *P. furiosus* also revealed the expression of *fbpA* (designated *suhB*) (18).

The *fbpA* gene (765 bp) was PCR amplified from chromosomal DNA of *P. furiosus* as described previously (21) with the primers BG977 (5'-GCGCGTCATGAAGCTTAAGTTCTG GAGGG [with the *Bsp*HI restriction site underlined], sense) and BG978 (5'-GCGCGGATCCCCTACTCCAGTAAGCTTA AAATTGTTTT [with the *Bam*HI restriction site underlined], antisense). The PCR product was digested with *Bsp*HI/*Bam*HI and cloned into *E. coli* XL1-Blue by use of an *Nco*I/*Bam*HI-digested pET24d vector according to established procedures and with 50  $\mu$ g of kanamycin/ml for selection. Subsequently, the resulting plasmid, pLUW558, was transformed into *E. coli* BL21(DE3).

**Overexpression and purification of FBPase.** An overnight culture of *E. coli* BL21(DE3) harboring pLUW558 was used as a 1% inoculum in 0.5 liter of Luria-Bertani medium with 50  $\mu$ g of kanamycin/ml. Gene expression was induced by adding 0.1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) at an optical density at 600 nm of 0.5. Growth was allowed to continue for 10 h at 37°C, and cells were harvested by centrifugation (2,200  $\times$  g for 20 min at 4°C) and resuspended in 10 ml of 50

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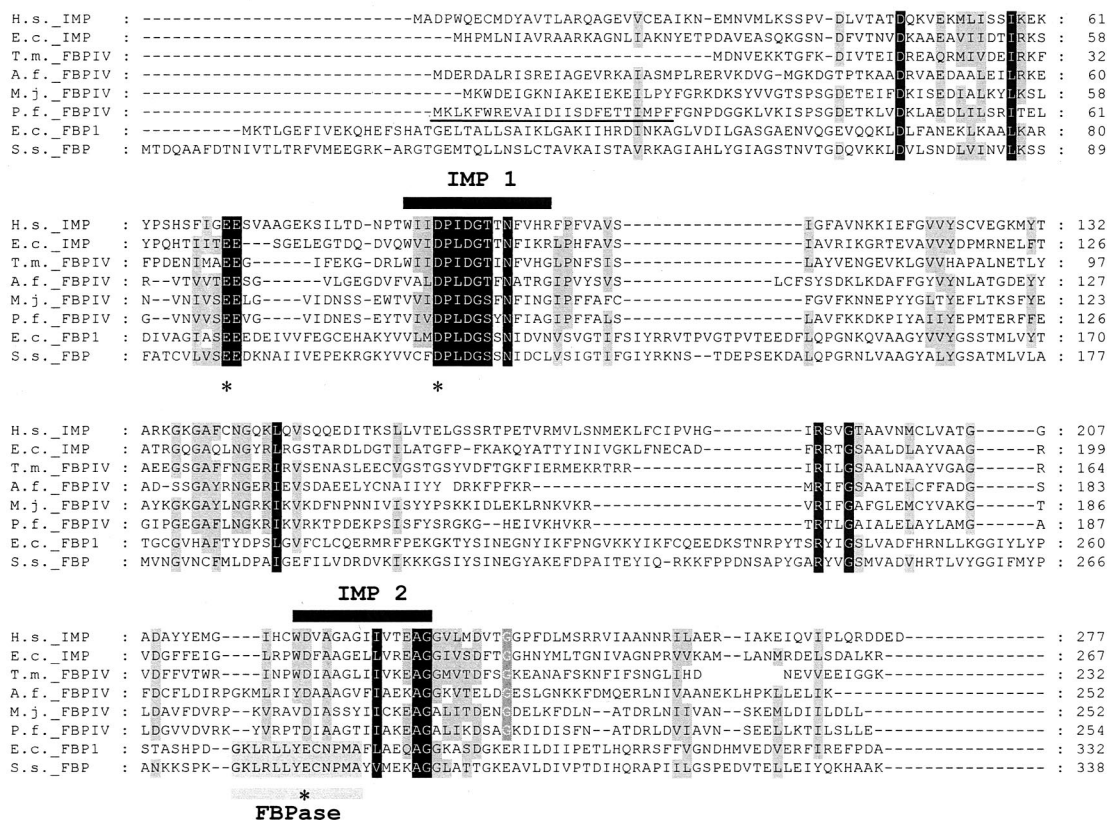


FIG. 1. Multiple sequence alignment of the deduced amino acid sequence of the *P. furiosus* FBpase with the sequences of its FBpase IV homologs and of I-1-Pases and FBpases from eukarya and bacteria. Abbreviations (accession numbers are given in parentheses): H.s. IMP, *Homo sapiens* I-1-Pase 1 (P29218); E.c. IMP, *E. coli* SuhB I-1-Pase (P22783); T.m. FBPIV, *T. maritima* TM1415 FBpase (O33832); A.f. FBPIV, *A. fulgidus* AF2372 FBpase (NP\_071195); M.j. FBPIV, *M. jannaschii* MJ0109 FBpase (Q57573); P.f. FBPIV, *P. furiosus* FBpase (AF453319); E.c. FBP1, *E. coli* FBpase (P09200); S.s. FBp, *Sus scrofa* FBpase (P00636). Gaps introduced by the alignment are indicated by dashes. Completely conserved regions are indicated by black boxes. Highly conserved regions are shaded gray. The IMP motifs are indicated with horizontal black bars above the alignments. The FBpase motif is indicated with a horizontal gray bar under the alignment. IMP 1 motif, [FWV]-x (0, 1)-[LIVM]-D-P-[LIVM]-D-[SG]-[ST]-x (2)-[FY]-x-[HKRNSTY]; inositol monophosphatase family signature 1 (PS00629). IMP 2 motif, [WV]-D-x-[AC]-[GSA]-[GSAPV]-x-[LIVACP]-[LIV]-[LIVAC]-x (3)-[GH]-[GA]; inositol monophosphatase family signature 2 (PS00630). FBpase motif, [AG]-[RK]-[LI]-x (1, 2)-[LIV]-[FY]-E-x (2)-P-[LIVM]-[GSA] (PS00124) (<http://www.expasy.ch/prosite>). Asterisks denote residues involved in the Li<sup>+</sup> binding site (24). The determined N-terminal amino acid sequence from the purified *P. furiosus* FBpase described in the text is underlined.

mM Tris-HCl buffer, pH 8.0. The cells were disrupted by French press treatment (100 MPa), and cell debris was removed by centrifugation (10,000 × g for 20 min at 4°C). The resulting cell extract was heat treated for 30 min at 80°C, and the precipitated proteins were removed by centrifugation (10,000 × g for 30 min at 4°C). The heat-stable cell extract was filtered through a 0.45-μm-pore-size filter and applied to a MonoQ HR 5/5 column (1 ml; Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The FBpase activity eluted at 0.37 M NaCl in a linear gradient of 0.0 to 1.0 M NaCl. Active fractions were pooled and concentrated 20-fold to a final volume of 100 μl by use of a filter with a 10-kDa cutoff (Microsep; Pall Filtron). The concentrated pool was loaded on a Superdex 200 HR 10/30 gel filtration column (24 ml; Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl buffer, pH 7.8, containing 100 mM NaCl. The elution pattern (not shown) suggested that the active configuration was a dimer (66.8 kDa) of two identical 33-kDa subunits, which is in good agreement with the results of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not

shown). The calculated size of the subunit was slightly smaller, i.e., 27.9 kDa. The purified enzyme was desalted in 50 mM Tris-HCl buffer, pH 8.0, by use of a filter with a 10-kDa cutoff (Microsep; Pall Filtron). From 2.7 g of cell paste consisting of *E. coli* BL21(DE3) containing pLUW558, a total of 27.7 mg of FBpase was purified to 95% purity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). To ensure that the detected activity corresponded to the *P. furiosus* FBpase, the N-terminal sequence of the purified enzyme was determined by the Edman degradation method to be Met-Lys-Leu-Lys-Phe-Trp-Arg-Glu-Val-Ala-Ile-Asp-Ile-Ile-Ser-Asp-Phe-Glu-Thr-Thr-Ile-Met-Pro-Phe, revealing that the obtained amino acid sequence exactly matched the N-terminal sequence of the translated *fbpA* from *P. furiosus* (Fig. 1). This indicates that the *P. furiosus* FBpase had been produced and purified successfully.

**Temperature dependence of FBpase.** For the determination of the temperature optimum, an appropriate amount of purified FBpase (6 to 30 ng) was incubated in 1-ml crimp-sealed vials containing 100 mM MOPS (morpholinepropanesulfonic

acid) buffer, pH 7.4, and 10 mM MgCl<sub>2</sub>. The vials were submerged in an oil bath at temperatures varying from 20 to 120°C and preheated for 2 min, and the enzyme reaction was initiated by the injection of 15 mM fructose-1,6-bisphosphate. At different times up to 15 min, the reaction was stopped by transferring the vials to a mixture of ice and ethanol. Aliquots were taken, and the amount of fructose-6-phosphate formed was determined spectrophotometrically by measuring the reduction of NADP<sup>+</sup> (340 nm) at room temperature in an assay with glucose-6-phosphate isomerase (EC 5.3.1.9) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), both from *Saccharomyces cerevisiae*. A linear increase in fructose-6-phosphate production over time was observed, indicating that no *P. furiosus* FBPase was inactivated during incubation. The *P. furiosus* FBPase showed maximal activity at approximately 100°C (data not shown).

In accordance with first-order inactivation kinetics (data not shown), the enzyme (18 µg/ml) lost 50% of its activity after incubation for 2 h at 100°C in 50 mM Tris-HCl buffer, pH 8.0. For the determination of the melting temperature, *P. furiosus* FBPase was dialyzed extensively against a 100 mM sodium phosphate buffer, pH 8.0, and diluted to 0.3 mg/ml in dialysis buffer. After being degassed for 10 min, the samples were analyzed against the dialysis buffer in a differential scanning microcalorimeter (VP-DSC; MicroCal) between 50 and 125°C at 0.5°C/min. Enzyme scans were corrected with a buffer-buffer baseline, and data were analyzed with the MicroCal Origin 5.0 SR2 software package. For FBPase, an apparent melting temperature of 107.5°C was determined (data not shown), which is in good agreement with the inactivation kinetics.

**Catalytic properties.** The kinetic parameters of the *P. furiosus* FBPase were determined discontinuously at 85°C by varying the concentration of fructose-1,6-bisphosphate (between 0.005 and 5 mM) and by measuring the release of inorganic phosphate at room temperature as described previously (8). The 0.2-ml assay mixture contained 50 mM Tris-HCl buffer (pH 8.0; room temperature), 10 mM MgCl<sub>2</sub>, and 0.4 µg of purified FBPase. At this temperature, the  $K_m$  and  $V_{max}$  of the *P. furiosus* FBPase with fructose-1,6-bisphosphate were  $0.32 \pm 0.03$  mM and  $12.2 \pm 0.1$  U/mg, respectively, resulting in a catalytic efficiency ( $k_{cat}/K_m$ ) of  $17.7 \text{ s}^{-1} \text{ mM}^{-1}$ . The determined affinity of the purified FBPase for fructose-1,6-bisphosphate is in good agreement with the previously determined  $K_m$  of 0.5 mM (75°C) in a *P. furiosus* extract (16). The kinetic parameters of the purified FBPase determined at 50°C were as follows: a  $K_m$  of  $0.31 \pm 0.06$  mM, a  $V_{max}$  of  $0.72 \pm 0.04$  U/mg, and a catalytic efficiency of  $1.12 \text{ s}^{-1} \text{ mM}^{-1}$ . Thus, the *P. furiosus* FBPase clearly is a thermoactive enzyme with affinities for fructose-1,6-bisphosphate at 50 and 85°C that are similar.

The specific activities of the *P. furiosus* FBPase for fructose-1,6-bisphosphate and related substrates were determined at 85°C in the standard assay that measures the release of inorganic phosphate. The 1-ml assay mixture contained 50 mM Tris-HCl buffer (pH 8.0; room temperature), 2.5 mM substrate, 10 mM MgCl<sub>2</sub>, and 0.02 mg of purified FBPase. At fructose-1,6-bisphosphate concentrations above 10 mM, the enzyme was subjected to substrate inhibition (data not shown). The highest activity was obtained with fructose-1,6-bisphosphate (12.2 U/mg). In addition, *myo*-inositol-1-phosphate, glucose-1-phosphate, and glycerol-2-phosphate could also be de-

TABLE 1. Substrate specificity of *P. furiosus* FBPase compared to that of *M. jannaschii* MJ0109<sup>a</sup>

Substrate	Relative activity (%)	
	<i>P. furiosus</i> FBPase	<i>M. jannaschii</i> MJ0109
Fructose-1,6-bisphosphate	100	100
Inositol-1-phosphate	7.5	61
Glycerol-2-phosphate	1.7	49
Glucose-1-phosphate	2.8	42

<sup>a</sup> Assays for *P. furiosus* FBPase were performed at 85°C as described in the text. The data from assays of MJ0109 performed at 85°C were obtained from Stec et al. (20). An activity of 100% corresponds to 12.2 and 15.2 U/mg for *P. furiosus* FBPase and MJ0109, respectively. Fructose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, PEP, 5'-AMP, 5'-ADP, and 5'-ATP could not be used as substrates by the *P. furiosus* FBPase.

phosphorylated by the enzyme, although the activities for these substrates were relatively low (1.7 to 7.5%) (Table 1). The recently described I-1-Pase/FBPase from *M. jannaschii* (MJ0109) also dephosphorylates these substrates but with a higher relative activity (42 to 61%) (20) (Table 1). The *P. furiosus* FBPase appeared to be a rather specific phosphatase, since fructose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, phosphoenolpyruvate (PEP), 5'-AMP, 5'-ADP, and 5'-ATP could not be used as substrates under the tested conditions.

The low I-1-Pase activity of the *P. furiosus* FBPase might be explained as follows. In thermophilic archaea and bacteria, several intracellular solutes are accumulated in response to osmotic and temperature stresses (15). One of these compatible solutes is di-*myo*-inositol phosphate (DIP), a solute that accumulates at supraoptimal growth temperatures in some thermophilic species (15, 17). In *P. furiosus*, temperatures above the growth optimum also lead to a significant increase of this compound (11). Two different routes for DIP synthesis are known: (i) in *Methanococcus igneus* (closely related to *M. jannaschii*), I-1-Pase activity is required to form *myo*-inositol, which acts as a precursor in DIP biosynthesis (4), and (ii) in *Pyrococcus woesei*, DIP is synthesized in a different way, without the *myo*-inositol-forming step (17). This latter pathway includes the coupling of two *myo*-inositol-1-phosphates without a preceding I-1-Pase-mediated dephosphorylation of one of the *myo*-inositol-1-phosphate moieties. Since *P. furiosus* is closely related to *P. woesei*, it is most likely that in *P. furiosus*, I-1-Pase activity is not required for DIP synthesis either, which would be in good agreement with the observed low activity of the *P. furiosus* FBPase on *myo*-inositol-1-phosphate.

**Effectors of FBPase.** The effect of inhibitors on the activity of the *P. furiosus* FBPase was investigated by adding cations and metabolites (0 to 100 mM) to the standard enzyme assay mixture (85°C) (Table 2). The enzyme has an absolute requirement for Mg<sup>2+</sup> (data not shown). The inhibition characteristics of the *P. furiosus* FBPase clearly differ from those of characterized eukaryal and bacterial FBPases as well as from those of the other presently characterized archaeal I-1-Pase/FBPase homologs. FBPase I from *E. coli* is very sensitive to AMP and PEP (1). FBPase II from *E. coli* is strongly inhibited by ATP and ADP, whereas AMP has no effect on the enzyme activity. Furthermore, FBPase II activity is enhanced in the presence of PEP (6). PEP also affects FBPase III activity, i.e., inhibition by



TABLE 2. Inhibitors of *P. furiosus* FBPase activity<sup>a</sup>

Effector	IC <sub>50</sub> (mM)
Li <sup>+</sup> .....	1
Ca <sup>2+</sup> .....	5
AMP.....	30
ADP.....	3
ATP.....	4
Glucose-6-phosphate.....	4
Fructose-6-phosphate.....	25
Pyruvate.....	60

<sup>a</sup> Enzyme assays were performed at 85°C as described in the text (10 mM fructose-1,6-bisphosphate). IC<sub>50</sub>, the concentration of the effector when activity of the *P. furiosus* FBPase was reduced by 50%. The addition of up to 100 mM Na<sup>+</sup>, K<sup>+</sup>, glucose, or PEP to the assay mixture had no effect on FBPase activity.

AMP is reduced when PEP is present (7). The *P. furiosus* FBPase was inhibited by ADP and ATP (and to some extent by AMP), but PEP (up to 100 mM) did not influence FBPase activity at all. Therefore, PEP presumably is not an important metabolite in the regulation of FBPase in *P. furiosus*. In addition, glucose-6-phosphate significantly reduced *P. furiosus* FBPase activity in vitro (Table 2).

Li<sup>+</sup> is generally a strong inhibitor of FBPase activity ( $K_i \sim 0.3$  mM) (24). Under the test conditions we used, Li<sup>+</sup> significantly reduced the *P. furiosus* FBPase activity (50% inhibitory concentration [IC<sub>50</sub>], 1 mM) (Table 2), whereas the addition of Na<sup>+</sup> and K<sup>+</sup> produced no effect. Previously, it was shown that I-1-Pases are also strongly inhibited by Li<sup>+</sup> (IC<sub>50</sub>  $\sim 0.3$  mM) (12). These enzymes have a structural fold similar to that of FBPases (27), and both are members of the sugar phosphatase superfamily (<http://scop.mrc-lmb.cam.ac.uk/scop>). Inhibition of mammalian I-1-Pase by Li<sup>+</sup> is of particular interest, since this enzyme is expressed in brain tissue and forms the main target in medical treatment of manic-depression (14). The mechanisms of the Li<sup>+</sup> inhibition of FBPases and IMPases are believed to be essentially the same: Li<sup>+</sup> binds at one of the metal binding sites, thereby retarding turnover or phosphate release (9, 24). The residues that constitute this metal binding site are conserved in lithium-sensitive I-1-Pase and in FBPase (Fig. 1). Remarkably, Li<sup>+</sup> does not have such a strong effect on the *T. maritima* (TM1415) and *M. jannaschii* (MJ0109) enzymes (IC<sub>50</sub>s, 100 mM for TM1415 and >250 mM for MJ0109), although residues constituting the Li<sup>+</sup> binding site are conserved (Fig. 1) (9). Minor variations will probably dis-

tinguish the inhibitory effect of Li<sup>+</sup> on the I-1-Pase from that on FBPase (9).

**Classification of FBPases.** Recently, a new classification of bacterial FBPases into three groups (FBPase I, FBPase II, and FBPase III) has been proposed (6). Eukaryal FBPases are orthologous to the bacterial FBPase I enzymes, since both contain typical FBPase domains (<http://www.expasy.ch>) and display no I-1-Pase activity (20). The typical FBPase domain is absent in the bacterial FBPase II and FBPase III enzymes (Table 3), suggesting that they are phylogenetically unrelated to FBPase I enzymes. Remarkably, typical I-1-Pase domains (IMP 1) are also present in the eukaryal FBPase and the bacterial FBPase I enzymes (<http://www.expasy.ch>). Bacterial and eukaryal I-1-Pases contain two specific domains (IMP 1 and IMP 2) and, together with the eukaryal FBPase and bacterial FBPase I enzymes, belong to the sugar phosphatase superfamily (<http://scop.mrc-lmb.cam.ac.uk/scop>). Comparison of the primary structure of the *P. furiosus* FBPase with the FBPase and IMP family signatures revealed that this enzyme contains both I-1-Pase domains (IMP 1 and IMP 2). No obvious FBPase domain could be detected in the *P. furiosus* sequence (Table 3) (Fig. 1). The *P. furiosus* FBPase is homologous to *M. jannaschii* MJ0109, *A. fulgidus* AF2372, and *T. maritima* TM1415, with all three enzymes having IMP 1 and IMP 2 domains present in their primary structures (Fig. 1) and possessing dual activity (i.e., FBPase and I-1-Pase activity) (20). Since these FBPases display limited sequence identity with both eukaryal and mesophilic bacterial FBPases (12 to 16% identity with FBPase I and 11 to 15% identities with FBPase II and FBPase III) but seem to be significantly related to the I-1-Pases (16 to 35% identity), we propose on the basis of sequence identity and substrate specificity that the *P. furiosus* FBPase and its homologs constitute a new FBPase family, type IV FBPases (FBPase IV), which is present in euryarchaeal and hyperthermophilic bacterial species and is potentially involved in gluconeogenesis. The presence of conserved domains (IMP 1) in type I and type IV FBPases and I-1-Pases, as well as the similar structural folds of these enzymes (20, 27), suggests that these enzymes share the same phylogenetic origin, as has been suggested previously (20, 27). It is tempting to speculate that the FBPase IV enzymes originally belonged to the I-1-Pase family and subsequently evolved to efficiently convert fructose-1,6-bisphosphate for function in gluconeogenesis.

**Nucleotide sequence accession number.** The *P. furiosus* *fbpA*

TABLE 3. Classification of phosphatases

Phosphatase class	Characteristic				
	Taxonomic range	Subunit size (kDa)	Oligomerization	Fold type	Sequence motif(s)
FBPase I	Eukarya, bacteria	~38	Tetramer	Sugar phosphatase	FBPase, IMP 1
FBPase II	Bacteria	~36	Dimer	Unknown	None
FBPase III	Bacteria	~76	Tetramer	Unknown	None
FBPase IV <sup>a</sup>	Archaea, HT bacteria	~28	Dimer <sup>b</sup>	Sugar phosphatase	IMP 1, IMP 2
I-1-Pase <sup>c</sup>	Eukarya, bacteria	~30	Dimer	Sugar phosphatase	IMP 1, IMP 2

<sup>a</sup> FBPase IV enzymes are at least present in the euryarchaea *P. furiosus* (AF453319), *Pyrococcus horikoshii* (PH1897), *Pyrococcus abyssi* (PAB0189), *Methanococcus jannaschii* (MJ0109), *Archaeoglobus fulgidus* (AF2372), *Methanosarcina barkeri* (MB1918), and *Methanobacterium thermoautotrophicum* (MTH871) and the hyperthermophilic (HT) bacteria *Thermotoga maritima* (TM1415) and *Aquifex aeolicus* (AQ1983).

<sup>b</sup> The *T. maritima* enzyme is an exception, having a tetrameric structure.

<sup>c</sup> Bacterial extragenic suppressor proteins (SuhB) are classified within the I-1-Pase family (3) and show I-1-Pase but not FBPase activity (20).

nucleotide and amino acid sequence data reported in this study have been submitted to GenBank under accession no. AF453319.

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