# SUPPORTING INFORMATION

## Phosphomannose isomerase/GDP-mannose pyrophosphorylase from *Pyrococcus furiosus*: a thermostable biocatalyst for the synthesis of guanidinediphosphate-activated and mannose-containing sugar nucleotides

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**Materials**. Enzymes and reagents used for the molecular biology procedures, DNA ladders, and deoxynucleotide triphosphates (dNTPs) were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Oligonucleotides for DNA amplification were synthesized by Sigma Genosys (Woodland, TX). Thermostable inorganic pyrophosphatase (IPP) from *Thermococcus litoralis* (EC 3.6.1.1, M0296S) was purchased from New England Biolabs as a 2000 U/mL 50% glycerol solution in Tris buffer (pH 8.0). Isopropylthiogalactoside (IPTG) was obtained from Labscientific, Livingston, NJ. Protein molecular weight standards were obtained from BioRad (Hercules, CA). The QIA Quick gel extraction kit was obtained from Qiagen (Valencia, CA) and the Zero Blunt PCR cloning kit

was purchased from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

**Bacterial Strains and Growth Conditions.** Genomic DNA from *Pyrococcus furiosus* (ATCC 43587D), obtained from the American Type Culture Collection (Manassas, VA), was used as the source for the cloning experiments described herein. Oneshot Top10 competent cells (Invitrogen, Carlsbad, CA), *Escherichia coli* XL-10Blue cells (Stratagene, La Jolla, CA), and PCR-Blunt vectors (Invitrogen, Carlsbad, CA) were used for direct cloning of PCR products. *E. coli* strain BL21 (DE3) RIPL (Stratagene, La Jolla, CA) was used in combination with the T7 expression system (pET21a vector; Novagen, Madison, WI.) for expression of the sugar nucleotidyltransferase gene. *E. coli* cells were grown on Luria-Bertani (LB, Sigma, St. Louis, MO) medium at 37 °C on an incubator shaker at 225 rpm. When required, antibiotics were added at the following concentrations to make the selective media: carbenicillin 50 μg/mL, kanamycin 50 μg/mL, chloramphenicol 25 μg/mL.

**General Methods.** Standard procedures to manipulate DNA, including plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation and transformation of *E. coli*, were performed by conventional methods (Sambrook 1989). The PCR was carried out in an Eppendorf Mastercycler gradient thermocycler (Eppendorf Scientific Inc. Westbury, NY). Protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Tris-HCl 10-20% gradients, Bio-Rad Laboratories, Hercules, CA). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined with the Bio-Rad protein assay kit according to the method of Bradford (Bradford 1976) using bovine serum albumin as the standard.

**PCR Amplification, Cloning, Expression and Purification of *P. furiosus* Enzyme.** Genomic DNA of *P. furiosus* (ATCC 43587D) was amplified by PCR synthesis using two oligonucleotide primers. The primers were designed in order to construct the G1P-TT expression plasmid. The forward primer, 5'-AAAC**CATATG**AAGACATTAATTCTTGCTGGAGG -3', contains an *Nde*I restriction site (in bold) and the reverse primer, 5'- AAA**CTCGAGCTAAGCCCTCTGGTAGTCGTCC**-3', contains a *Xho*I restriction site (in bold) and were synthesized from the putative (Robb 2001) mannose-6-phosphate isomerase/mannose-1-phosphate guanylyltransferase (manC) gene of *P. furiosus*. The amplification reaction mixture contained standard *Pfu*DNA polymerase buffer, 375 μM of dNTPs, 3 ng of each primer, 4 ng of total genomic DNA and 2.5 units of *Pfu* DNA polymerase. The cycling parameters of 94 °C for 2 min 40 sec followed by 30 cycles of 94 °C for 30 s, 56 °C for 45 s and 72 °C for 2 min 15 s, with a final elongation step of 72 °C for 15 min. The amplified DNA, after agarose gel electrophoresis (1%) was purified using QIAquick Gel Extraction kit and subcloned into a ZeroBlunt vector using ZeroBlunt PCR cloning kit, and was transformed into Oneshot Top10 and *E. coli* XL10 competent cells to check the correct insert. The resulting construct was then digested with *Nde*I and *Xho*I and was ligated to a pET21a vector containing a C-terminal histidine tag sequence (Novagen, Madison, WI) and previously digested with the same restriction enzymes. Aliquots of the ligation mixture were transformed into competent *E. coli* BL21 (DE3) RIPL cells. Transformants were selected at 37 °C grown on LB medium supplemented with carbenicillin. The freshly transformed cells containing the desired plasmid were grown in LB until the optical density at 600 nm of the cell culture reached 0.5-0.6. Enzyme production was initiated by the addition of IPTG (1 mM) and the culture was incubated at 37 °C for the

additional 3 h. Cells were harvested by centrifugation at 3600 x g for 10 min at 25 °C. The enzyme was purified essentially at 4 °C unless otherwise stated. The cells were disrupted by sonication (Fisher model 100 Sonic Dismembranator, Fisher Scientific, Pittsburgh, PA), after which unbroken cells and debris were removed by centrifugation (30 min at 10,000 x g). The supernatant was then heated at 100 °C for 5 min and the precipitated portion was removed by centrifugation at 12,000 x g for 20 min. The cleared lysate was then purified by metal chelate chromatography by following the recommended procedures provided by Novagen. The purified protein was concentrated and dialyzed into the Tris-HCl buffer (50 mM, pH 7.5) using a Microcon Centrifugal Filter Device, MWCO 10 kDa (Millipore, Billerica, MA). The protein was analyzed by SDS-PAGE analysis. No protein band was detected in SDS-PAGE of an *E. coli* cell extract harboring a pET21a vector without the manC insert. In order to construct a truncated mutation of the PMI-GMP the C-terminal PMI domain was deleted by PCR amplification of the manC gene using the forward primer described above and a separately designed reverse primer 5'-AAACTCGAG GCG TTA CCA TTC TCG TCC TTT TGA AG-3' with a *Xho*I restriction site incorporated (underlined). The PCR product was then cloned and expressed in *E. coli* BL21 (DE3) RIPL cells following the method described above. The enzymatic activity, substrate specificity, kinetic properties, and other biochemical functions were determined following the methods described else where.

**Mass Spectrometry.** A Shimadzu LCMS 2010 quadrupole mass spectrometer (Shimadzu Scientific Instruments, Columbia, MD) equipped with an electrospray ionization (ESI) source was used in negative ion mode. The capillary temperature and the spray voltage were kept at 220 °C and 4.5 kV, respectively. The instrument was calibrated by direct infusion of polyethylene glycol (PEG) 200, 600, 1000, (1.5 µL/L, 2 µL/L, and 15 µL/L, respectively) and raffinose (50 mg/L) in water/methanol (1:1, v:v) containing ammonium acetate (0.19 mM), 0.1% formic acid and 0.1% acetonitrile. For sample analysis the solvent acetonitrile/water/triethylamine, (35/65/0.2) was constantly infused into the ion source at 250 µL/min by the attached Shimadzu HPLC pump and the samples were injected (30 µL) via the auto sampler adapted to fit two 96-well plates. A preliminary MS chromatogram was obtained by scanning from 50-700 *m/z*. To increase the signal to noise ratio, the instrument was set for selected ion monitoring (SIM) mode and all relevant *m/z* ions were monitored for further analysis of the enzymatic reactions. Inclusion of an Agilent Extend C18 column (2.1 x 50 mm, Agilent, Palo Alto, CA) in the system further increased the signal to noise ratio and decreased the appearance of sodium ion adducts by 10-15% without separation of reaction components. Postrun software (LCMS Postrun version 2.02, Shimadzu Scientific Instruments, Columbia, MD) was used to analyze the data from the ESI-MS chromatogram. Peaks were integrated to determine the relative intensity of each ion species monitored as compared to an internal standard.

**Enzyme Assays.** The nucleotidyltransferase activity of the bifunctional *P. furiosus* enzyme was determined, according to the method described previously (Zea and Pohl 2004), by the formation of GDP-mannose from GTP and of mannose 1-phosphate (Man1P) in the direction of GDP-mannose biosynthesis. The enzymatic reaction was initiated by the addition of Man1P (5 mM) to a reaction mixture of 50 µL containing Na-phosphate buffer (25 mM, pH 7.5), inorganic pyrophosphatase (IPP, 0.2 U), purified enzyme solution (10 µL), $MgCl_2$ (5 mM), and GTP (5 mM). Before adding Man1P the reaction components were incubated at 80 °C for 5 min. The reaction was carried out at 80 °C for 5 min and 15 µL of the reaction mixture was quenched by the addition of 30 µL of 70% methanol/water containing AMP (3 mM) as an

internal standard. The quenched solutions were centrifuged 10 min at 10,000 x g to precipitate the protein. Aliquots of the reaction mixtures were diluted with 135 μL of acetonitrile (acetonitrile/water/triethylamine, 35/65/0.2). These samples (5 μL) were subjected to analysis via ESI-MS to determine the amount of GDP-mannose formed. One unit of enzyme activity is defined as one micromole of GDP-man was formed per minute. Detection of phosphomannose isomerase activity of the enzyme was performed at 30°C in 0.5 mL of 25 mM Tris-HCl buffer (pH 7.0) containing 5 mM $Co^{2+}$, 5 mM mannose-6-phosphate, 5 mM NADP, 10 U phosphoglucose isomerase and 10 U phosphoglucose dehydrogenase and the appropriate amount of purified manC according to the published method (Wu, 2002). The reduction of NADP+ was followed by monitoring of UV absorption at 340 nm.

**Optimal Activity Determination of the Enzyme.** The optimal activity for the *P. furiosus* enzyme was measured at 80 °C between pH 4.0 to pH 9.6 using 50 mM acetate, phosphate, and Tris-HCl buffer. The optimal temperature was measured at pH 7.5 between 30 °C to 100 °C. Relative acceptance of the enzyme to a number of sugar-1-phosphates and NTPs (UTP, dTTP, GTP, ATP and CTP), effects of divalent cations on catalytic conversion and $Mg^{2+}$ ion concentrations were determined in Tris buffer (25 mM, pH 7.5) and at 80 °C.

**Kinetic Analysis.** The values for $K_M$ and $V_{max}$ were derived from enzymatic reactions run in triplicate and determined from the initial rates of ADP-, CDP-, GDP-, dTDP-, and UDP-mannose or GDP-glucose formation using ESI-MS. The enzymatic reaction was initiated by the addition of Man1P (25-500 μM) or glucose-1-phosphate to obtain a reaction mixture containing Phosphate buffer (25 mM, pH 7.5), inorganic pyrophosphatase (0.2 U), manC (2.5 x $10^{-3}$ U), and NTP (400 μM) with a final volume of 50 μL. Reactions were carried out at 80 °C for 5 min and 30 μL of reaction mixture was quenched by addition of 30 μL of 70% methanol/water containing AMP (6 μM) as an internal standard. The quenched solution was centrifuged and diluted as described above. These samples (30 μL) were subjected to analysis via ESI-MS to determine the amount of NTP-mannose or GDP-glucose formed. Calibration curves for determining the initial activities for nucleotidyltransferase activity of the enzyme were developed following the methods described previously (Zea, 2004). All kinetic data was fitted with the non-linear regression algorithm in GraphPad Prism version 4 (GraphPad Software, San Diego, CA). Error bars on the graphs represent the standard deviation of three independent averaged data points.

**Multiple Sequence Alignment.** A multiple sequence alignment was performed using BLAST (www.ncbi.nih.gov). Motifs were defined as the regions with at least 11 strictly conserved residues among 30 consecutive positions (Bork, 1996).

**manC Catalyzed Synthesis of NDP-Sugars and HPLC.** To synthesize GDP-sugars and NDP-mannoses, one equivalent (2 to 3 mg) of sugar-1-phosphate and 1.5 equivalents of NTPs along with the other reaction components in Na-phosphate buffer (pH 7.5) including inorganic pyrophosphatase (IPP, 2 U) were incubated with purified manC at 80 °C for 1 h. The formation of product was confirmed by ESI-MS by monitoring of the NDP-sugar. The yield determined by ESI-MS was calculated as the difference of the amount of sugar phosphate remaining in the reaction and the initial amount then divided by the initial amount of substrate used in the reaction (Mizanur, 2004). The incubation mixture was loaded on a 5μm SAX phenosphere HPLC column (250 x 10 mm) fitted with a security guard cartridge

(Phenomenex, CA) after ultrafiltration using a YM-10 membrane (Millipore corporation, Bedford, MA) to remove any protein. The NDP-sugars were eluted with 150 mM ammonium-phosphate buffer (pH 6.0) at ambient temperature and monitored at 254 nm. Fractions collected were further analyzed by ESI-MS to check the identity of the products, then pooled and lyophilized. Retention times were as follows: GDP-Man (5.4 min), UDP-Man (4.2 min), ADP-Man (5.7 min), CDP-Man (4.6 min), dTDP-Man (4.5 min), GDP-Glc (5.7 min), GDP-Gal (5.4 min), GDP-GlcN (5.7 min), GDP-GlcNAc (5.3 min), GDP-Fuc (6.3 min), whereas all the nucleotide triphosphates eluted sometime after 7 min. Using similar reaction conditions that include inorganic pyrophosphatase, 50 mg scale synthetic reactions were also carried out containing one equivalent of sugar-1-phosphate (Man1P, Glc1P, GlcN1P and GlcNAc1P) and 1.5 equivalents of GTP with manC.

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**Table S1**. Effects of divalent cations on the activity of the *P. furiosus* enzyme.

| *Metal ions* | *Relative activity (%)* |
|---|---|
| None[a] | 0 |
| $Mg^{2+}$ | 100 |
| $Ca^{2+}$ | 30 |
| $Cu^{2+}$ | 90 |
| $Mn^{2+}$ | 25 |
| $Co^{2+}$ | 33 |
| $Zn^{2+}$ | 81 |

[a]All of the reaction components were incubated without any divalent cation at 80 °C.

**Table S2.** Substrate specificity of the enzyme.

| Sugar-1-phosphate | ATP | CTP | GTP | dTTP | UTP |
|---|---|---|---|---|---|
| Man1P | + | + | + | + | + |
| Glc1P | + | + | + | + | + |
| Gal1P | Nd | Nd | + | Nd | Nd |
| GlcN1P | + | + | + | Nd | + |
| GlcNAc1P | Nd | Nd | + | Nd | Nd |
| Fuc1P | Nd | Nd | + | Nd | Nd |

Nd: Not detected by mass spectrometry.

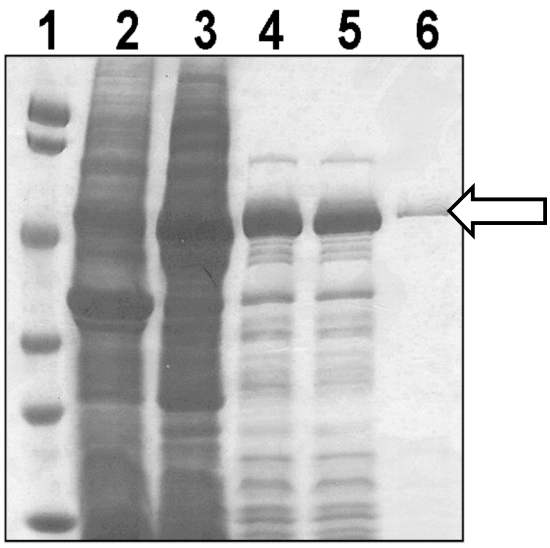


**Figure S1.** SDS-PAGE after Coomassie staining of the purification process for the 6x *His* tagged *P. furiosus* manC expressed in *E. coli* using a Ni-NTA spin column. Lanes: 1, molecular weight standards in kDa (descending order): phosphorylase *b* (108), bovine serum albumin (90), ovalbumin (50.7), carbonic anhydrase (35.5), soybean trypsin inhibitor (28.6), lysozyme (21.2); 2, cell pellet after centrifugation and removal of supernatant; 3, crude extract from *E. coli*; 4, crude extract after heating at 100 °C for 5 min; 5, column wash; 6, column elution containing the purified protein. Arrow indicates purified manC used for enzymatic reactions.

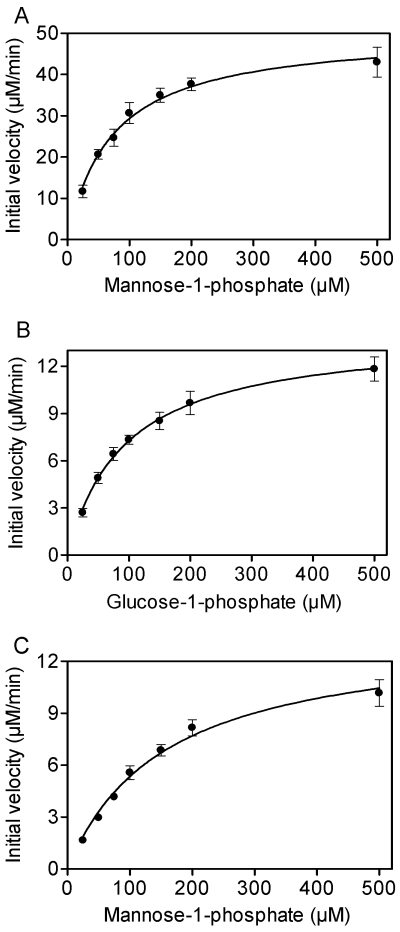


**Figure S2.** Michaelis-Menten plots for the recombinant *P. furiosus* enzyme in the direction of NDP-sugar synthesis for Man1P with GTP (A), Glc1P with GTP (B), and Man1P with UTP. Error bars on the graphs represent the standard deviation of three independent averaged data points.

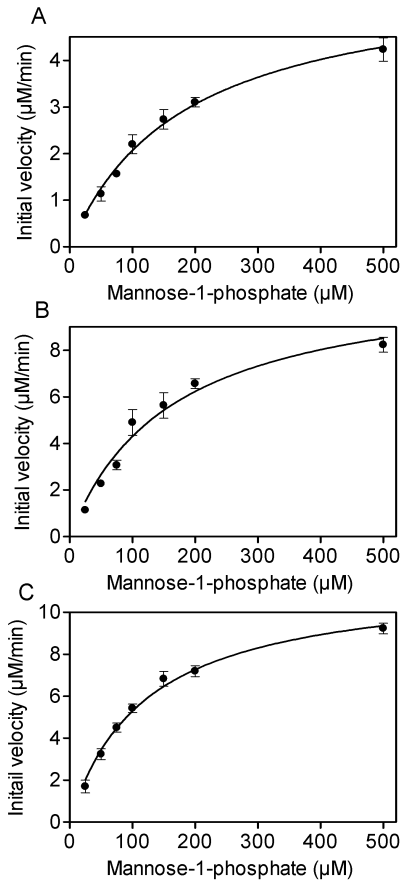


**Figure S3.** Michaelis-Menten plots for recombinant *P. furiosus* enzyme in the direction of NDP-sugar synthesis for Man1P with dTTP (A), CTP (B), and ATP (C) respectively. Error bars on the graphs represent the standard deviation of three independent averaged data points.

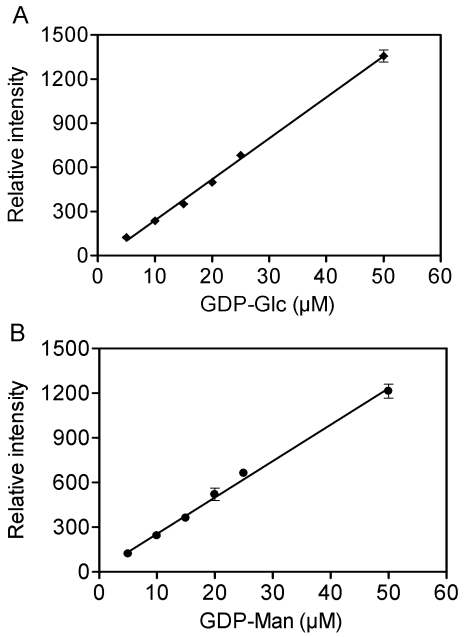


**Figure S4.** Calibration curves for the relative intensity of (A) GDP-Glc (*m/z* = 604) and (B) GDP-Man (*m/z* = 604).

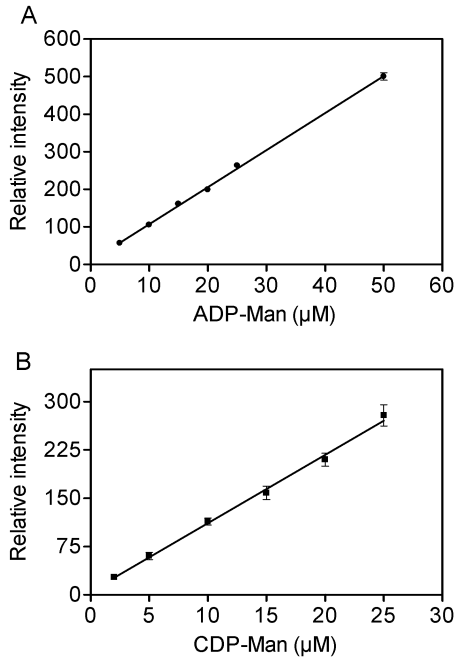


**Figure S5.** Calibration curves for the relative intensity of (A) ADP-Man (*m/z* = 588) and (B) CDP-Man (*m/z* = 564).

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a.1 MKTLILAGGKGTRLWPLSRELMPKQFIKLFS     ESLFQKTVKRALY.[1].S.[1].PDEIYVITNKEYRFRVLDDL
b.1 MKTLILAGGKGTRLWPLSRELMPKQFIKVFS.[1].KSLFQKTVERALI.[1].S.[1].PKEIFVVTNKEYRFRVLDDL
c.6 IKSIILAGGSGTRLWPLSREMYPKQFLKFGD     TSLFQETVLRCLE.[1].S.[1].ISEIFVVTNEAQKFFVIGQI
d.7 LIPCIVSGGSGTRLWPVSRESMPKPFMRLAD.[1].QSLLQKTFLRIAG.[1].P.[1].VARLLTVTNRDLLFRTLDDY
e.1 MKTLILAGGKGTRLWPLSREAMPKQFIKVFS.[1].RSLFQKTVERALL.[1].S.[1].PKEIFIVTNKEYKFRVLDDL
f.1 MKALILAGGKGTRLWPLSREAMPKQFIKVFS.[1].RSLFQKTVERALI.[1].S.[1].PKEIFVVTNKEYRFRVLDDL
g.2 MKALILAGGSGERFWPLSTPETPKQFLKLFG.[1].KSLMRWTFERVLE.[1].M.[1].PKDVIVVTHKDYVERTKKEL
h.4 LLPVIMAGGAGSRLWPLSRALYPKQFLALTS.[1].LTMLQETLLRLDG     L.[1].HLAPLVICNEEHRFIIAEQL
i.1 MKILILSGGKGTRLWPLSRENFPKQFIRLFS.[1].HSLFQETVKRAKK.[1].A.[1].DEDIVVITGEKYEWIIRSEL
j.6 LYPVVMAGGSGSRLWPLSRVLYPKQFLCLKG.[1].LTMLQTTICRLNG     V.[1].CESPVVICNEQHRFIVAEQL

a.68  EE.[2].IS.[5].IILEPEAKNTLPAICLGVKAAGE.[2].FAVLPSDHLIKA.[2].EYLNAFRSAEKLSE.[1].YI
b.69  NE.[2].VN.[5].ILLEPVGKNTLPAIYWGLKVIDE.[6].VAVLPSDHAIDV.[2].NYIEAFKKAEKLAE.[1].YL
c.73  KE.[2].YS.[5].VLIEPEGKNTLPAIFFGMKEIEQ.[6].VGVFSSDHVLDR     AAMATIFSAEKLTS.[1].YL
d.75  RA.[2].RS.[5].LLLEPVGRNTAPAIAAAALHVQE.[7].LLILPADHLIRD.[2].AFAAAVAEARGLAA.[2].YL
e.69  NE.[2].LK.[5].ILLEPVGKNTLPAIYWGLKVIDE.[6].VAVLPSDHAIKV.[2].NYMEAFKKAEKLAE.[1].YL
f.69  NE.[2].LK.[5].ILLEPVGKNTLPAIYWGLKVIND.[6].VAVLPSDHAIEV.[2].SYMEAFKKAEKLAE.[1].YL
g.70  PE.[2].DE.[1].IIAEPMKKNTAPACFIGTKLADD.[3].VLVLPADHRIPD.[2].KFWKTVKKALDALE.[3].GL
h.71  RQ     KN.[5].IVLEPVGRNTAPAIALAALRATM.[6].LLVLAADHVIQD.[2].AFIRAVQRAEPLAE.[2].KL
i.69  EE.[2].AN.[3].VVVEPEGRNTAPAIALGVKYLIH.[8].VLVLPSDHLIKD.[2].KFVEAVKKGEKYAK.[2].YI
j.73  RQ     LN.[5].IILEPAGRNTAPAIALAALAAKR.[8].MLVLAADHVIAD.[2].AFRAAVRNAMPYAE.[2].KL

a.135 VTFGITPTRPHTGYGYIKPGK.[ 5].FEVEQFKEKPSRELAEEYVSKG     YLWNSGMFVFDSKVFVEELKELAPE
b.140 VTFGIKPTKPHTGYGYIKPGE.[ 6].FLVDEFKEKPDLETAKRYVENG     YYWNSGMFMFRTTLFMEEARKHAPD
c.142 VTFGVVPAFPHTGYGYIKAAE.[ 5].YRVSEFREKPDFETAQKYIEEG     CLWNSGMFLFETRLFFEEVKKHAPS
d.148 VTFGITPERAETGFGYIEQGA.[ 5].FRVARFVEKPDQATAQSYLDSG.[1].YLWNAGMFCFQAATVLQELERHAPE
e.140 VTFGIKPTKPHTGYGYIKPGE.[10].YLVDEFKEKPDLETAKKYVENG     YYWNSGMFMFRTSVFMEEARKHAPE
f.140 VTFGIKPTKPHTGYGYIKPGE.[10].YLVDEFKEKPDLETARKYVENG     YYWNSGMFMFKVSVFMEEARKHSPD
g.136 FTFGIVPTRPETGYGYIEIGE.[ 6].HKVAQFREKPDLETAKKFVESG.[1].FLWNSGMFLWKAREFIEEVKVCEPS
h.141 VTFGIVPKSPETGYGYIRQGK.[ 6].YQVAAFVEKPDLITAERYLASG.[1].YYWNSGMFVFKASRYLQELDLHRPD
i.141 VLFGEKPTYPETGYGYIRLNG.[ 6].YEIEKFEEKPDYEKAKEYVSDG.[1].HFWNCGIFLFTLDRIVKDYTQLMPE
j.145 VTFGIVPDLPETGYGYIRRGE.[10].FEVAQFVEKPNLETAQAYVASG.[1].YYWNSGMFLFRAGRYLEELKKYRPD

a.208 .[1].AKVLE.[ 2].EEAYKQIPEASFDYAILEKSGRVAVVPIKTFWSDLGNFDSIYEVMEKDE.[5].KSE.[2].IP
b.214 .[2].KAFEE.[ 4].EEAYELAPEISVDYGIMEKTDKAAVVPLNTYWNDLGSFDAVYEALQKDE.[5].EVR.[6].IN
c.215 .[1].FACFE.[ 5].DEIYACVDNVSIDYGIMEKSDRVAVVKLDQKWSDLGNFAAIYDELEKDS.[5].HEC.[2].ML
d.222 .[2].IAARA.[19].AGAFAEAPDISVDYALMERSDKVAVVPCSIGWSDIGSWQALRELSAADE.[5].RGE.[1].VL
e.218 .[1].VRAFE.[ 5].EEIYELVPEISVDYGIMEKTDKAAVVPLNTYWNDLGSFDAVYEALDKDE.[5].QVA.[6].IN
f.218 .[1].VKAFE.[ 5].EEIYELAPEISVDYGIMEKTNKAAVVPLNTYWNDLGSFDAVYEALEKDE.[5].HVT.[6].IN
g.211 .[2].ENLKD.[ 9].KKAYEKVPSISVDYAVMEKSKKVRVVKADFEWSDVGNWSSVREIEGYTE     ESD.[2].IL
h.216 .[2].AACKQ.[15].EEAFSSCPDESIDYAVMEKTSDAVVPLDAQWNDVGCWSALWEINTKDD.[5].RGD.[1].LI
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j.224 .[2].DACEK.[15].EEAFLACPEESVDYAVMERTADAVVPMDAGWSDVGSWSSLWEISAHTA.[5].HGD.[1].IN

a.277 VDSENNLVITQ.[1].LTALIGLRDLIVIDTDDALLV.[2].
b.290 VDSKNNLILTE.[1].LTATVGVEDLIIVDTGDALLV.[2].
c.287 LNSDGNLVYSK.[3].IVSLIDIKDMVIVDTSDALLI.[2].
d.308 HDVSNCYIDSP.[2].LVGAVGVHDLIIVDTPDALLV.[2].
e.294 INSRNNLILTE.[1].LTATVGVEDLIIVDTGDALLV.[2].
f.294 VDSRNNLVLTE.[1].LTATVGVEDLVIIDTGDALLV.[2].
g.283 VDSDRVFVKTH.[2].PIAVVGLSDVIVIDTPNGILI.[2].
h.298 EDTNNSYVYSQ.[2].LIATVGINDLVIVETKDAILV.[2].
i.286 MDTECSLLLSQ.[3].LIACIGLEDFVVVGTEDATLI.[2].
j.306 HKTENSYVYAE.[2].LVTTVGLKDLVVVQTKDAVLI.[2].
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**Figure S6.** Mannose-1-phosphate guanylyltransferase (manC) domain of the bifunctional enzyme from *P. furiosus* and its amino acid sequence similarity with other homologous enzyme. a, *Archaeoglobus fulgidus* DSM 4304 (NP_069926); b, *Pyrococcus furiosus* DSM 3638 (NC_003413); c, *Methanosarcina acetivorans* C2A (NP_618652); d, *Pseudomonas aeruginosa* PAO1 (NP_250922); e, *Pyrococcus abyssi* GE5 (NP_126910); f, *Pyrococcus horikoshii* OT3 (NP_142848); g, *Thermotoga maritima* MSB8 (NP_228839); h, *Yersinia pestis* CO92 (NP_406586); i, *Aquifex aeolicus* VF5 (NP_213412); j, *E. coli* (P24174). Identical amino acids are indicated by bold-faced letters.

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a.278DSENNLVIT.[1].RLTALIGLRDLIVIDTDDALLVARRGEAEKVREVYRLLAEKGDKAVEVHRTAHRPWGSYTVLEENK
b.291DSKNNLILT.[1].RLTATVGVEDLIIVDTGDALLVAKKGETQKVKEVYKKLKEENDERAIVHRTAYRPWGSYTVLEEGE
c.298 DTEGCFVSS.[2].MVTSLVGVRDLVVVAEKDAILVADRSRCGDVRKMVEILRSKGRPQAEWHASSHRPWGSYRVLEASD
d.307 DVEGSYIRS.[2].RLVAVAGLCNVVVVATDDAVLVIDRGKVQDVKQIVERLKKANRDEHALHSTVHRPWGHYRGIDRGE
e.298 DSHNCLVHG.[2].KLVSVIGLEDIVVVETKDAMMIAHKDRVQDVKHVVKDLDAQGRSETQNHCEVYRPWGSYDSVDMGG
f.297 DCKNTYAYG.[1].RLIAMVGLENVVVVETDDAVLVGHRDRIQEVKEVVSQIKSAGRSEATWHRKVYRPWGAYDSIDMGQ
g.301 KSQNNYVFS.[2].RLVSLLGVDNLVVIETKDAILVADKSKVQDIKKIVESIKEQGRTEHFCHREVYRPWGKYDSIDHAE
h.300 NADNCYLHA.[2].GLVTAVGVKDLIVVQTKDAVLVANTNCVQDVKKIVEKIKLENRHEHITHREVYRPWGKYDSIDFGE
i.295 STTNSLVRA.[2].RLVATVGVNNLVVIETADAVLIMDKDQSQDVKKIVSRIKAEGRQEHMHHTTVHRPWGTYQTVDLGD
j.297 DTNNSYIYS.[2].RLVATVGINDLIIVETKDALLVANKNKVQSVKEIVGQLKLGSRLEYLQHKEVYRPWGSHDAIAEGV

a.354 SYKIKRITVKPKKRLSLQRHYHRSEHWVVVKGTARIVVDGNEILLRSGESTFVPAGAIHRIENPGKIPLEIIEIQIGEYL
b.367 RYKIKRITVLPGKRLSLQLHYHRSEHWVVVRGTAKVRVGDKEFILRPGESTFIPAGVIHRLENPGKVVLEVIETQIGEYL
c.375 GFQVKRITVAPGGRLSLQKHRHRAEHWVVVRGCARVTVDDTVADYRESEHIFIPLGAIHRLENPGNDDVELIEVQLGSYL
d.384 RFQVKRIVVQPGERLSLQMHHHRAEHWIVVTGTALVTRGAETFLLHENESTYIRAGQTHRLENPGKVPLHLIEVQSGGYL
e.375 RFQVKHITVKPGARLSLQMHHHRAEHWIVVSGTAQVTCDDKTFLLTENQSTYIPIASVHRLANPGKIPLEIIEVQSGSYL
f.373 RFQVKRITVKPGATLSLQMHHHRAEHWIVVSGTAEVTRGEEVLLLTENQSTYIPLGVTHRLKNPGKLPLELIEVQSGSYL
g.378 RYQVKRITVKPGQKLSIQMHHHRSEHWIVVNGTAKIHKGKESFLLTENQSTYIPLGEIHALENPGKVPLELIEVQSGSYL
h.377 RYQVKRITVKPGEGISEQQHYHRAEHWIIVAGTAKITIKGEVKILTENESVYIPVGVKHCLENPGKIALELIEVRSGAYL
i.372 RHQVKRIMVKPGEKLSVQMHHHRAEHWVVVSGTAKVQNGEREILLTENESTYIPVGVVHALENPGKIPLELIEVQSGSYL
j.374 RYHVQHVTIKPGQRTATQIHHHRAEHWVVVSGTARVYRDNESYLVTENESTYIAVGVAHSIENPGKLPLEIIEVRTGSYL

a.434 EEDDIERFED
b.447 GEDDIVRIED
c.455 GEDDIVRLED
d.464 GEDDIVRFED
e.455 GEDDIERLED
f.453 GEDDIVRFED
g.458 GEDDIVRFED
h.457 GEDDIVRFSD
i.452 GEDDIVRFSD
j.454 EEDDIVRIEH
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**Figure S7.** Mannose-6-phosphate isomerase domain of the bifunctional enzyme from *P. furiosus* and its amino acid sequence similarity with other homologous enzymes. a, *Archaeoglobus fulgidus* DSM 4304 (AAB90149); b, *Pyrococcus furiosus* DSM 3638 (NC_003413); c, *Methylobacterium extorquens* (AAC46167); d, *Rhodospirillum rubrum* (BAA02175); e, *Pseudomonas aeruginosa* (P07874); f, *Xanthomonas campestris* pv. *campestris* (P29956); g, *Acinetobacter calcoaceticus* (CAA57100); h, *Salmonella enterica* (Q01410); i, *Vibrio cholerae* O139 (AAC46245); j, *Yersinia enterocolitica* (AAC60775). Identical amino acids are indicated by bold-faced letters.

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                a
eco  9    VVMAGGTGSRLWPLSRELYPKQFLQLSGDNTLLQTTLLRLSGLSCQKPL-VITNEQHRFV  67
pfu  4    LILAGGKGTRLWPLSRELMPKQFIKVFSNKSLFQKTVERALIFSKPKEIFVVTNKEYRFR  63

eco  68   VAEQLREI--NKLNGNIILEPCGRNTAPAIAISAFHALKRNPQE--DPLLLVLAADHVIA  123
pfu  64   VLDDLNEIGVNIPEENILLEPVGKNTLPAI----YWGLKVIDESFGDSIVAVLPSDHAID  119

eco  124  KESVFCDAIKNATPIANQGKIVTFGIIPEYAETGYGYIERGELSVPLQGHENTGFYYVNK  183
pfu  120  VNENYIEAFKKAEKLA-ENYLVTFGIKPTKPHTGYGYIKPGEKI------SNLGFL-VDE  171
              b
eco  184  FVEKPNRETAELYMTSGNHYWNSGIFMFKASVYLEELRKFRPDIYNVCEQVASSSYIDLD  243
pfu  172  FKEKPDLETAKRYVENG-YYWNSGMFMFRTTLFMEEARKHAPDVVKAFEEG---------  221

eco  244  FIRLSKEQFQDCPAESIDFAVMEKTEKCVVCPVDIGWSDVGSWQSLWDISLKSKTGDVC-  302
pfu  222  --KTIEEAYELAPEISVDYGIMEKTDKAAVVPLNTYWNDLGSFDAVYEALQKDENGNAVE  279

eco  303  ----KGDILTYDTKNNYIYSESALVAAIGIEDMVIVQTKDAVLVSKKSDVQHVKKIVEML  358
pfu  280  VRGFKAKYINVDSKNNLILTER-LTATVGVEDLIIVDTGDALLVAKKGETQKVKEVYKKL  338
                                                    c
eco  359  KLQQRTEYISHREVFRPWGKFDSIDQGERYKVKKIIVKPGEGLSLRMHHHRSEHWIVLSG  418
pfu  339  KEENDERAIVHRTAYRPWGSYTVLEEGERYKIKRITVLPGKRLSLQLHYHRSEHWVVVRG  398
                       d
eco  419  TAKVTLGDKTKLVTANESIYIPLGAAYSLENPGIIPLNLIEVSSGDYLGEDDIIRQKERY  478
pfu  399  TAKVRVGDKEFILRPGESTFIPAGVIHRLENPGKVVLEVIETQIGEYLGEDDIVRIEDDY  458

eco  479  K  479
pfu  459  Q  459
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**Figure S8.** Amino acid sequence alignment of *P. furiosus* manC with an *E. coli* homolog. pfu: *P. furiosus;* eco: *E. coli.* The identical amino acids are indicated in bold. Highlighted amino acids are different domains essential for the activity of the manC. : [a], pyrophosphorylase signature; [b], GMP active site; [c], cation binding site; [d], PMI active site.