

Pathway for the Synthesis of Mannosylglycerate in the Hyperthermophilic Archaeon *Pyrococcus horikoshii*

BIOCHEMICAL AND GENETIC CHARACTERIZATION OF KEY ENZYMES*

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Nuno Empadinhas^{‡§¶}, Joey D. Marugg^{§||}, Nuno Borges^{‡ ¶¶}, Helena Santos^{‡**},
and Milton S. da Costa[§]

From the [‡]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Rua da Quinta Grande 6, Apartado 127, 2780-156 Oeiras, Portugal, the [§]Departamento de Bioquímica, Universidade de Coimbra, 3000 Coimbra, Portugal, and ^{||}Nestlé Research Center, CH-1000 Lausanne 26, Switzerland

The biosynthetic pathway for the synthesis of the compatible solute α -mannosylglycerate in the hyperthermophilic archaeon *Pyrococcus horikoshii* is proposed based on the activities of purified recombinant mannosyl-3-phosphoglycerate (MPG) synthase and mannosyl-3-phosphoglycerate phosphatase. The former activity was purified from cell extracts, and the N-terminal sequence was used to identify the encoding gene in the completely sequenced *P. horikoshii* genome. This gene, designated PH0927, and a gene immediately downstream (PH0926) were cloned and overexpressed in *Escherichia coli*. The recombinant product of gene PH0927 catalyzed the synthesis of α -mannosyl-3-phosphoglycerate (MPG) from GDP-mannose and D-3-phosphoglycerate retaining the configuration about the anomeric carbon, whereas the recombinant gene product of PH0926 catalyzed the dephosphorylation of mannosyl-3-phosphoglycerate to yield the compatible solute α -mannosylglycerate. The MPG synthase and the MPG phosphatase were specific for these substrates. Two genes immediately downstream from *mpps* and *mppg* were identified as a putative bifunctional phosphomannose isomerase/mannose-1-phosphate-guananyltransferase (PH0925) and as a putative phosphomannose mutase (PH0923). Genes PH0927, PH0926, PH0925, and PH0923 were contained in an operon-like structure, leading to the hypothesis that these genes were under the control of an unknown osmosensing mechanism that would lead to α -mannosylglycerate synthesis. Recombinant MPG synthase had a molecular mass of 45,208 Da, a temperature for optimal activity between 90 and 100 °C, and a pH optimum between 6.4 and 7.4; the recombinant MPG phosphatase had a molecular mass of 27,958 Da and optimum activity between 95 and 100 °C and between pH 5.2 and 6.4. This is the first report of the characterization of MPG synthase and MPG phosphatase and the elucidation of a pathway for the synthesis of mannosylglycerate in an archaeon.

The vast majority of microorganisms capable of osmotic adjustment to environmental alterations in salt levels accumulate small molecular weight organic solutes, termed compatible solutes or osmolytes, to maintain a positive turgor pressure and to protect enzymes from desiccation (1, 2). The accumulation of compatible solutes can be accomplished by specific uptake or by *de novo* synthesis of osmolytes. The uptake of organic solutes such as glycine betaine and trehalose, among other solutes, from the environment is preferred because it is energetically favorable (3). Many organisms, however, synthesize their own compatible solutes because specific solutes in the environment may not be freely available or do not fulfill the prerequisites of an osmolyte.

Ectoine, hydroxyectoine, glycine betaine, trehalose, and glutamate are probably among the most common compatible solutes of bacteria and archaea (4). However, slightly halophilic thermophilic and hyperthermophilic bacteria and archaea generally accumulate compatible solutes that are rare or unknown in mesophilic organisms. Moreover, these osmolytes usually have a negative charge that is neutralized by the accumulation of potassium (5–7). The archetypal compatible solutes of thermophiles and hyperthermophiles such as di-*myo*-inositol phosphate (DIP),¹ dimannosyl-di-*myo*-inositol phosphate, diglycerol phosphate, mannosylglyceramide, and mannosylglycerate (MG) have not been found in mesophilic bacteria and archaea. Mannosylglycerate appears to be a very common compatible solute in thermophilic and hyperthermophilic organisms, namely in *Pyrococcus furiosus*, the slightly halophilic *Thermococcus* spp., *Aeropyrum pernix*, *Thermus thermophilus*, *Rhodothermus marinus*, and *Petrogala miotherma* (6, 8–11). Furthermore, some of the compatible solutes of thermophiles and hyperthermophiles, of which MG deserves special mention, have been shown to protect enzymes *in vitro* against thermal denaturation and could also have an important role in thermoprotection of cell components *in vivo* (12, 13).

Basic knowledge of the biosynthesis of specific compatible solutes is needed to understand the mechanisms underlying the events leading to salt and thermal tolerance, from water stress sensing to maintenance of the appropriate intracellular levels of compatible solutes (14). The pathways for the synthe-

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** To whom correspondence should be addressed: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Rua da Quinta Grande 6, Apartado 127, 2780-156 Oeiras, Portugal. Tel: 351-214469800; Fax: 351-214428766; E-mail: santos@itqb.unl.pt.

¹ The abbreviations used are: DIP, di-*myo*-inositol phosphate; MG, α -mannosylglycerate; IPTG, isopropyl- β -D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Bis-Tris, 2-[bis(hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; MPG, α -mannosyl-3-phosphoglycerate; MPG synthase, mannosyl-3-phosphoglycerate synthase; MPG phosphatase, mannosyl-3-phosphoglycerate phosphatase; ORF, open reading frame; CAPSO, 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid; M1P-GT/PMI, mannose-1-phosphate-guananyltransferase/phosphomannose isomerase.

sis of osmolytes in thermophilic and hyperthermophilic organisms have only recently begun to be examined. Two pathways for the biosynthesis of MG exist in the thermophilic bacterium *R. marinus*. One pathway involves the single step conversion of GDP-mannose and D-glycerate to MG by mannosylglycerate synthase. An alternative pathway was also detected in *R. marinus* that leads to the synthesis of the phosphorylated intermediate, mannosyl-3-phosphoglycerate which, in turn, is converted to MG by a phosphatase (15). We deemed it important to investigate whether a similar strategy would hold in hyperthermophilic archaea, and the slightly halophilic archaeon *Pyrococcus horikoshii* (16) was selected for this purpose.

We identify the genes involved in the synthesis of MG in *P. horikoshii* and elucidate a pathway for the biosynthesis of this compatible solute, which involves a mannosyl-3-phosphoglycerate synthase (MPG synthase) and a specific mannosyl-3-phosphoglycerate phosphatase (MPG phosphatase). In addition, the genes for the synthesis of MG in *P. horikoshii* were cloned and overexpressed in *Escherichia coli*, and the recombinant enzymes were characterized in detail.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Conditions

The type strain of *P. horikoshii* (JCM 9974) was obtained from the Japanese Collection of Microorganisms (JCM), Saitama, Japan. The organism was cultivated as follows: the medium contained per liter, 5.0 g of peptone, 1.0 g of yeast extract, 25.0 g of NaCl, 1.0 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 40.0 mg of KH_2PO_4 , 19.6 ml of magnesium salts solution (180.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 140.0 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ /liter), 1.0 ml of solution A (4.0 g of trisodium citrate, 9.0 g of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2.5 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g of $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.3 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.15 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /liter), 2.0 ml of solution B (56.0 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 25.0 g of NaBr, 16.0 g of KCl, 10.0 g of KI, and 4.0 g of $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ /liter), 2.0 ml of solution C (50.0 g of K_2HPO_4 , 7.5 g of H_3BO_3 , 3.3 g of $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 0.15 g of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 0.005 g of Na_2SeO_3 /liter), and 20.0 ml of solution D (0.1 g of citric acid, 0.75 g of nitrilotriacetic acid, 0.06 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 14.5 g of KCl, and 32.0 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ /liter). The final pH was adjusted to 7.0. The medium was gassed with N_2 and sterilized by autoclaving. Sterile sulfur (3.0 g/l) was added to the medium after cooling to 90 °C. Cultures were grown in a 5-liter fermentor at 98 °C with continuous gassing of N_2 and stirred at 80 rpm.

To examine the effect of osmotic stress on the synthesis of intracellular solutes by *P. horikoshii*, 15–45 g of NaCl/liter was added to the medium. Biomass production for enzyme purification was carried out in medium containing 4.5% NaCl (w/v). Cell growth was monitored by measuring the turbidity at 600 nm.

E. coli XL1-Blue was used as host for expression vectors pTRC99A, pKK223-3 (Amersham Pharmacia Biotech), and for a plasmid isolated from *E. coli* strain BL21-CodonPlus (Stratagene) that carries extra tRNA genes for codons commonly found in archaea but rarely used by *E. coli*. This organism was grown in YT medium, at pH 7.0 and 37 °C, containing 10.0 g of tryptone, 5.0 g of yeast extract, and 5.0 g of NaCl/liter. Ampicillin was added at a final concentration of 100 µg/ml for selection of plasmids pKK223-3 and pTRC99A. Chloramphenicol was added to a final concentration of 20 µg/ml for selection of plasmid carrying the tRNA genes. IPTG was obtained from Roche Molecular Biochemicals and added at a final concentration of 1 mM.

Extraction and Determination of Intracellular Solute

Cells of *P. horikoshii* were harvested during mid-exponential phase of growth by centrifugation (7000 × g, 10 min, 4 °C) and washed once with a NaCl solution identical in concentration to that of the medium where the cells were grown. Cell pellets were extracted twice with boiling 80% ethanol as described previously (6). For solute quantification by ^1H NMR, the final pH of the samples was adjusted to ~8 by the addition of NaOH . The protein content of the cells was determined by the Bradford assay (17) after lysis with 1 N NaOH (100 °C, 10 min) and neutralization with 1 N HCl.

Preparation of Cell-free Extracts

Cells were harvested by centrifugation (7000 × g, 10 min, 4 °C) during the late exponential phase of growth. The cell pellet was resuspended in Tris-HCl (20 mM (pH 7.6)) containing MgCl_2 (5 mM), DNase

I (10 µg), and protease inhibitors, phenylmethylsulfonyl fluoride (80 µg), leupeptin (20 µg), and antipain (20 µg) per ml of the suspension. Cells were disrupted in a French press, followed by centrifugation (130,000 × g, 1 h, 4 °C). The supernatant was dialyzed against 20 mM Tris-HCl (pH 7.6) to remove endogenous mannosylglycerate (MG) and other low molecular weight compounds prior to measuring enzyme activities and purification procedures.

Enzyme Assays

To determine the combined activity of mannosyl-3-phosphoglycerate synthase (MPG synthase)/mannosyl-3-phosphoglycerate phosphatase (MPG phosphatase) in the cell extracts, the reaction mixture contained 2.5 mM GDP-mannose (Sigma) and 2.5 mM D-3-phosphoglycerate (sodium salt, Sigma) in 20 mM Tris-HCl (pH 7.6) with 10 mM MgCl_2 . The reaction mixtures were incubated at 90 °C for 30 min, and the MG produced was quantified by ^1H NMR after freeze-drying and dissolving in $^2\text{H}_2\text{O}$. Formate was used as an internal concentration standard. This protocol was also used to examine the kinetic parameters, temperature, and pH optima, the effect of NaCl, KCl, and divalent cations, and thermal stability of the recombinant MPG synthase, but in this case MPG was the final product that was quantified by ^1H NMR.

To detect the presence of MPG synthase, during the purification of the native enzyme, the same procedure was followed, but after incubation at 90 °C, unless otherwise stated, for 30 min, the reaction mixture was cooled to 37 °C, and 2 units of alkaline phosphatase (Sigma) was added. The mixture was incubated for an additional 30 min at 37 °C, and the formation of MG was visualized by TLC.

The activity of the purified recombinant MPG phosphatase was measured with 2 mM MPG in 20 mM Tris-HCl (pH 7.6) containing 10 mM MgCl_2 at 98 °C by monitoring the release of inorganic phosphate using the spectrophotometric method described by Ames (18). All enzyme parameters of MPG phosphatase were examined using this reaction mixture, unless otherwise stated.

MPG used for these assays was obtained from a reaction catalyzed by pure recombinant MPG synthase with 12.5 mM GDP-mannose, 12.5 mM D-3-phosphoglycerate as substrates, in 20 mM Tris-HCl (pH 7.6) and 10 mM MgCl_2 at 98 °C for 15–20 min. Quantification of MPG was carried out by incubating an aliquot of the reaction mixture with MPG phosphatase for 15–20 min to ensure complete dephosphorylation of MPG, in 20 mM Tris-HCl (pH 7.6) with 10 mM MgCl_2 . Inorganic phosphate released was quantified by the Ames method. The concentration of MPG in the reaction mixture was calculated from the concentration of inorganic phosphate produced. All reactions were stopped by freezing in liquid nitrogen.

Purification of Mannosyl-3-phosphoglycerate

MPG was partially purified from the reaction mixtures described above. Samples were loaded onto a QAE-Sephadex A-25 column previously equilibrated with 5.0 mM sodium bicarbonate (pH 8.0), and the elution was performed with 1 bed volume of the same buffer, followed by a linear gradient of 5.0 mM to 1 M NaHCO_3 . The eluted fractions were analyzed for carbohydrate by the method of Dubois *et al.* (19). MPG was eluted at 0.5 M NaHCO_3 . For a second chromatographic step, a column of activated Dowex 50W-X8 resin was used, and the elution was carried out with distilled water. Subsequently, the fractions were pooled and degassed under vacuum, and the pH was raised to 3.5 with 1.0 M KOH. Samples were lyophilized and dissolved in $^2\text{H}_2\text{O}$ prior to NMR analysis.

NMR Spectroscopy

The identification of the phosphorylated intermediate, α -mannosyl-3-phosphoglycerate, was accomplished using ^{13}C , ^{31}P , and ^1H NMR spectroscopy. Spectra were recorded on Bruker AMX300 or DRX500 spectrometers (9, 11). For quantification of substrates and products of enzyme reaction mixtures, ^1H NMR spectra were acquired on a Bruker AMX300 spectrometer with a broadband inverse probe head with pre-saturation of the water. Spectra were acquired with a repetition delay of 45 s, and formate was used as an internal concentration standard. Proton, carbon, and phosphorus chemical shifts are relative to 3-(trimethylsilyl)propanesulfonic acid, methanol (at 49.3 ppm), or 85% H_3PO_4 , respectively.

Analysis of MG Formation by Thin Layer Chromatography

TLC was performed on Silica Gel 60 plates (Merck) with a solvent system composed of chloroform, methanol, acetic acid, and water (30:50:8:4, v/v). MG was visualized by spraying with α -naphtholsulfuric acid solution followed by charring at 120 °C (20). Authentic standards of

MG, mannose, guanosine, and GDP-mannose were used for comparative purposes.

Purification of Native MPG Synthase

Native enzyme was purified by fast protein liquid chromatography (Amersham Pharmacia Biotech) at room temperature from *P. horikoshii* cell extracts.

Ion-exchange Chromatography—The cell-free extract was applied to a column (XK50/30, bed volume 250 ml) packed with DEAE-Sepharose fast flow equilibrated with Tris-HCl (20 mM (pH 7.6)). All the purification steps were carried out at pH 7.6. Elution was carried out with a two-step linear NaCl gradient (0.0–0.6 and 0.6–1.0 M) in the same buffer. MPG synthase activity was found in the fraction eluting between 0.4 and 0.5 M NaCl. Active fractions were pooled, concentrated, and equilibrated with 20 mM Tris-HCl. The sample was applied to a Q-Sepharose fast flow column equilibrated with the same buffer. Elution was carried out with a five-step discontinuous NaCl gradient (0.2, 0.4, 0.6, 0.8, and 1.0 M). The fractions eluting at 0.4 and 0.6 M NaCl contained MPG synthase activity. Fractions with MPG synthase activity were pooled, concentrated, and equilibrated with 20 mM Tris-HCl. This sample was applied to a 6-ml Resource Q column. Elution was carried out with a linear NaCl gradient (0.0–1.0 M). Fractions eluted between 0.29 and 0.34 M contained MPG synthase activity.

Gel Filtration Chromatography—The active fractions were pooled and concentrated by ultrafiltration (30-kDa cutoff) and were applied to a Superdex 200 column equilibrated with 0.2 M NaCl in 50 mM Tris-HCl. The active MPG synthase fractions were concentrated by ultrafiltration (10-kDa cutoff) and applied to a Superose column equilibrated with 0.2 M NaCl in 50 mM Tris-HCl. The purity of the active fraction was assessed by SDS-PAGE (21). The sample was blotted on polyvinylidene difluoride membranes followed by N-terminal amino acid sequencing at Microchemical Facility, Emory University School of Medicine, GA.

DNA Techniques, Analysis, Cloning, and Functional Overexpression of *mpps* (PH0927) and *mppg* (PH0926) in *E. coli*

Most DNA manipulations followed standard molecular techniques and procedures (22). *P. horikoshii* chromosomal DNA was isolated according to Marmur (23). Based on the N-terminal amino acid sequence of the purified MPG synthase, a corresponding open reading frame (ORF), designated PH0927, was identified from the *P. horikoshii* OT3 complete genome sequence (24). ORF sequences surrounding PH0927 were also screened for homologies with known phosphatase genes using the (T)FASTA (25) and (T)BLAST (26) algorithms. PCR amplifications were carried out in a PerkinElmer Life Sciences GeneAmp PCR System 2400 in reaction mixtures (50 μ l) containing 100 ng of *P. horikoshii* DNA, 100 ng of each primer, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 50 mM KCl, 1 unit of *Pwo* DNA polymerase, and 0.2 mM of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech). The mixture was preincubated for 5 min at 95 °C and then subjected to 30 cycles of denaturation at 95 °C for 1 min. Annealing was performed at 60 °C for 1 min, and primer extension was at 72 °C for 2 min. The extension reaction in the last cycle was prolonged for 5 min. Amplification products were purified from agarose gels (Bio-Rad).

Based on the complete gene sequence, *mpps* was amplified by the forward primer (5'-GCGCATGGTCTAGAAAGCTCC-3') and adding a recognition sequence for *Nco*I (underlined) including the ATG start codon. The reverse primer (5'-GCGCTGCAGTCATAGCTCAAACCTCAG-3') was constructed by adding an additional *Pst*I recognition sequence (underlined) directly behind the TGA stop codon. Gene *mppg* was amplified with the forward primer (5'-GCGGAATTCATGATTAGTTAATATTC-3') constructed with an additional *Eco*RI recognition sequence (underlined) immediately upstream of the ATG start codon, and the reverse primer (5'-GCGCTGCAGTCATTTGATCACCTCC-3') with an additional *Pst*I recognition sequence (underlined) directly behind the TGA stop codon. The PCR products were purified after digestion with *Nco*I and *Pst*I for *mpps* and with *Eco*RI and *Pst*I for *mppg* and ligated into corresponding sites of expression vectors pTRC99A and pKK223-3 to obtain plasmids pMPGS and pMPGP, respectively. Each construction was transformed into *E. coli* XL1-Blue cells previously transformed with the plasmid carrying tRNA genes for rare codons. Host cells containing pMPGS or pMPGP were grown to mid-exponential growth phase ($A_{600} = 0.6$), induced with IPTG, and grown further for 6–8 h. Cells were harvested and treated as described above for the preparation of cell-free extracts.

Purification of Recombinant MPG Synthase

E. coli cell extracts containing MPG synthase were incubated for 20 min at 80 °C to denature the majority of the host proteins and centri-

fuged (25000 \times g, 15 min, 4 °C). MPG synthase activity assay was performed as described above, and the enzyme was purified.

Ion-exchange Chromatography—The supernatant was applied to a DEAE-Sepharose fast flow column (XK50/30), equilibrated with 20 mM Tris-HCl (pH 7.6). Elution was carried out with a two-step linear NaCl gradient (0.0–0.6 and 0.6–1.0 M) in the same buffer. MPG synthase activity was found in the fraction eluting between 0.25 and 0.5 M NaCl. Active fractions were pooled, concentrated, and equilibrated to 20 mM Tris-HCl. The sample was applied to a 6-ml Resource Q column and eluted with a linear NaCl gradient (0–1 M). Fractions with activity eluted between 0.2 and 0.6 M. Purity of the samples was determined by SDS-PAGE. Three different pools with different degrees of purity resulted from this purification step.

Gel Filtration Chromatography (Superose 12)—The purest pool was concentrated by ultrafiltration (10-kDa cutoff). Fractions were applied to a gel Superose 12 column equilibrated with 0.35 M NaCl in 50 mM Tris-HCl (pH 7.6) and eluted with the same buffer. Active fractions were subjected to dialysis against 20 mM Tris-HCl (pH 8.0).

Anion-exchange Chromatography (Mono Q)—The sample was loaded onto a Mono Q column that was eluted by a linear gradient of NaCl (0–1 M). The fraction with MPG synthase eluted between 0.55 and 0.6 M NaCl.

Purification of Recombinant MPG Phosphatase

Extracts for the purification of MPG phosphatase, as well as the assay for enzyme activity were as described above.

Ion-exchange Chromatography—The MPG phosphatase-containing supernatant was applied to a DEAE-Sepharose fast flow column as described for the recombinant MPG synthase. Elution was carried out with a two-step linear NaCl gradient (0.0–0.5 and 0.5–1.0 M). MPG phosphatase eluted between 0.2 and 0.35 M NaCl. Active fractions were concentrated, dialyzed against 20 mM Tris-HCl (pH 7.9), and loaded onto a Mono Q column that was eluted by a linear gradient of NaCl (0.0–1.0 M). The fraction with MPG phosphatase eluted between 0.25 and 0.3 M NaCl.

Characterization of Recombinant MPG Synthase and MPG Phosphatase

All biochemical and kinetic parameters for these enzymes were determined using the assay conditions described above. The temperature profiles for activity of MPG synthase and MPG phosphatase were determined between 30 and 108 °C. The effect of pH on MPG synthase activity was determined at 98 °C in 50 mM BisTris/propane buffer (pH 6.5–9.5) and 50 mM CAPSO (pH 7.0–10.0). The effect of pH on MPG phosphatase activity was determined at 98 °C in 50 mM acetate buffer (pH 3.4–5.4), 50 mM BisTris/propane buffer (pH 6.5–9.5), and CAPSO (pH 7.0–10.0). All pH values were measured at room temperature (25 °C); pH values at 98 °C were calculated using the conversion factor $\Delta pK_a/\Delta T$ °C = -0.015 for BisTris/propane and -0.018 for CAPSO. Enzyme thermostabilities were determined at 98 °C by incubating enzyme solutions (0.5 mg/ml) in 20 mM Tris-HCl (pH 7.6). At appropriate times, samples were withdrawn and immediately examined for residual activities at 98 °C.

Kinetic parameters for MPG synthase were determined in reaction mixtures containing GDP-mannose (0.1–5.0 mM) plus D-3-phosphoglycerate (5 mM) or GDP-mannose (5 mM) plus D-3-phosphoglycerate (0.1 to 5.0 mM). Reaction mixtures for the determination of the kinetic parameters of MPG phosphatase contained MPG (0.1–2.0 mM). Samples of MPG synthase and MPG phosphatase reactions were pre-heated for 3 and 2 min, respectively, and all reactions were initiated by the addition of the enzyme preparation. Kinetic parameters for all substrates were determined at 98 °C. All experiments were performed in duplicate. Values for V_{max} and K_m were determined from Hanes plots.

RESULTS

Effect of NaCl Concentration of the Medium on Growth and Solute Accumulation by *P. horikoshii*—This organism had a behavior illustrative of slightly halophilic organisms, requiring 1.5–5.0% NaCl in the culture medium for growth, with an optimum for growth of about 2.5% NaCl (Fig. 1). Cells grown in medium with 1.5% NaCl were enlarged, compared with cells grown at higher NaCl concentrations, and the cell yield was significantly lower than after growth under the other conditions. At the lowest salinity examined for compatible solute accumulation (2.5% NaCl), the total pool of solutes was low. At this salinity MG was the major compatible solute (0.11

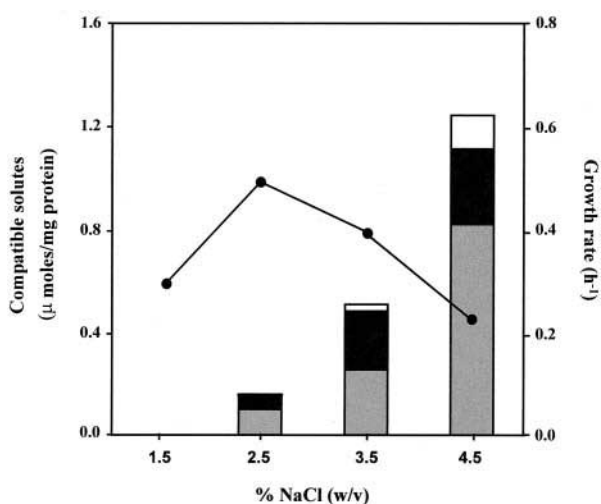


FIG. 1. Effect of the NaCl concentration of medium on the growth rate (●) and on the accumulation of compatible solutes (■, mannose-1-phosphate; ■, di-myoinositol phosphate; □, trehalose) during growth of *P. horikoshii* at the optimum growth temperature, 98 °C.

μmol/mg protein) compared with trace levels of DIP (0.04 μmol/mg protein). An increase of the salt concentration of the medium to 3.5% NaCl caused an increase in MG concentration to 0.24 μmol/mg protein and of DIP to 0.21 μmol/mg protein, whereas trehalose remained vestigial. At the highest salinity examined (4.5% NaCl), there was a large increase in MG levels to 0.84 μmol/mg protein, without a concomitant alteration in the levels of DIP. An increase in the trehalose concentration was also observed in this medium.

Synthesis of MG in Cell Extracts—From an array of experiments using GDP-mannose, UDP-mannose, and ADP-mannose as possible sugar donors and D-3-phosphoglycerate and D-glycerate as the sugar acceptors, we only detected the formation of MG in cell extracts from GDP-mannose and D-3-phosphoglycerate. The specific activity for MG production in *P. horikoshii* cell extracts was 6.1 nmol/min-mg protein. MPG synthase/MPG phosphatase activities could not be measured independently in cell extracts nor was a phosphorylated intermediate detected by TLC, but the hypothesis of a two step pathway was confirmed during the purification of MPG synthase, because the phosphatase activity was separated after the Resource Q step. The analysis of the reaction mixture by TLC after this step showed a compound that did not co-migrate with standard MG. Incubation of this reaction mixture with alkaline phosphatase led to the formation of a compound that co-migrated with MG.

Purification of Native MPG synthase and Identification of *mpps* and *mppg* Genes—MPG synthase was purified in five chromatographic steps, the final preparation containing three bands on SDS-PAGE (results not shown). The N-terminal sequence analyses of the three bands led to the identification of three separate ORFs in the *P. horikoshii* genome, one of which (*PH0927*) coded for a 45-kDa protein enclosing a conserved domain for family 2 glycosyltransferases in its sequence (NCBI, PSI-BLAST) (Fig. 2), the size of which was in agreement with one of the bands in the denaturing gel. Therefore, this protein was considered the most likely candidate for MPG synthase. Moreover, the analysis of the sequences surrounding ORF *PH0927* revealed two ORFs (*PH0925* and *PH0923*) putatively identified as mannose-1-phosphate-guanylyltransferase/phosphomannose isomerase (M1P-GT/PMI, EC 2.7.7.22/EC 5.3.1.8) and phosphomannomutase (EC 5.4.2.8), respectively. The presence of genes putatively related to the synthesis of mannose-containing compounds near *PH0927* strengthened our hypoth-

esis that this was the MPG synthase gene. Additionally, these findings also indicated *PH0926* as the best candidate for a MPG phosphatase, which was further supported by the presence in its sequence of a conserved domain common to trehalose-6-phosphate phosphatases (Fig. 2).

Cloning, Functional Overexpression of *mpps* and *mppg* in *E. coli*, and Purification of Recombinant Enzymes—PCR amplification of *mpps* and *mppg* from genomic DNA of *P. horikoshii* yielded products with the expected gene sizes. For overexpression in *E. coli*, the PCR-amplified *mpps* and *mppg* were separately cloned under the control of the strong inducible *trc* promoter (pTRC99A) and *tac* promoter (pKK223-3), respectively. The sequence of the insert for *mppg* (*PH0926*) was identical to that of the native ORF. However, the cloning of *mpps* (*PH0927*) required the introduction of a *NcoI* site in the coding region, resulting in a substitution of leucine at position 2 to valine in the recombinant gene product.

Activity assays carried out in *E. coli* cell extracts revealed MPG production by MPG synthase clones and MPG dephosphorylation by MPG phosphatase clones but not by the negative control *E. coli* XL1-Blue (pTRC99A or pKK223-3) cell extracts. SDS-PAGE analysis of cell extracts from *E. coli* XL1-Blue (containing pMPGS and pMPGP clones) grown with IPTG induction showed extra bands of 45 and 28 kDa, respectively, that were not observed in cell extracts from *E. coli* XL1-Blue with empty vectors. The specific activities of MPG synthase and of MPG phosphatase in crude extracts of *E. coli* XL1-Blue were 356 and 162 nmol/min-mg protein at 98 °C, respectively. Heat treatment of cell extracts at 80 °C for 20 min resulted in extensive purification of the 45 kDa (MPG synthase) and the 28 kDa (MPG phosphatase) proteins. The specific activity of MPG synthase in heat-treated cell extracts of *E. coli* XL1-Blue (pMPGS) was 10.1 μmol/min-mg protein at 98 °C. Specific activity of MPG phosphatase in heat-treated cell extracts of *E. coli* XL1-Blue (pMPGP) was 3.3 μmol/min-mg protein at 98 °C. The purity of recombinant MPG synthase and MPG phosphatase was judged by SDS-PAGE (Fig. 3).

Catalytic Properties of MPG Synthase—Nine sugar nucleotides, namely ADP-mannose, GDP-mannose, UDP-mannose, ADP-glucose, GDP-glucose, UDP-glucose, TDP-glucose, UDP-galactose, and ADP-ribose, were used as possible sugar donors, and six 3-carbon compounds (glycerol, D-3-phosphoglycerate, D-2-phosphoglycerate, L-glycerol-3-phosphate, 2,3-diphospho-D-glycerate, and phosphoenolpyruvate) were used as sugar acceptors. Of these, only GDP-mannose and D-3-phosphoglycerate formed MPG (results not shown). The unequivocal identification of the reaction product as α-mannosyl-3-phosphoglycerate (MPG) was achieved by standard one- and two-dimensional NMR spectroscopy (Table I). In particular, the α-configuration was established from the measurement of the coupling constant between the anomeric carbon and the directly bound proton ($J = 171.8$ Hz).

MPG synthase exhibited Michaelis-Menten kinetics and the K_m values for the substrates are shown in Table II. The activity of MPG synthase in the absence of Mg^{2+} was 46% that in the presence of this divalent cation, but NaCl and KCl, in the concentration range of 50 to 300 mM, inhibited enzyme activity (Table II). At 40 °C the activity of the enzyme was undetectable, and maximal activity of the enzyme was reached between 90 and 100 °C (Fig. 4). At 108 °C MPG synthase still had 25% of maximal activity. Within the pH range examined (5.4–9.0), the activity of the enzyme at 98 °C was maximal between pH 6.4 and pH 7.4 (Fig. 5). At 98 °C, the optimal temperature for growth of *P. horikoshii*, the half-life for MPG synthase activity was 16 min (Fig. 6).

Catalytic Properties of MPG Phosphatase—Several sugar

TABLE I
NMR data of mannosyl-3-phosphoglycerate

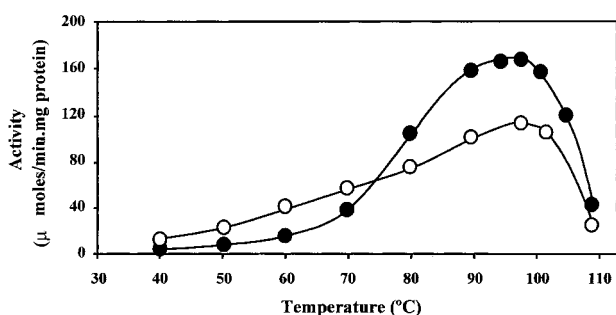
Moiety	¹³ C NMR			¹ H NMR			³¹ P NMR	pK _a ^b	
	δ	¹ J _{CH}	J _{CP}	δ	³ J _{HH}	J _{HP}	δ		
	ppm	Hz	Hz	ppm	Hz	Hz	ppm		
Mannose									
C-1	98.9	171.8		4.88	³ J _{2'3'} = 1.7				
C-2	70.7	148.7		4.08	³ J _{2'3'} = 3.4				
C-3	70.9	144.9		3.96	³ J _{3'4'} = 9.8				
C-4	67.9	145.9		3.59	³ J _{4'5'} = 9.8				
C-5	73.3	148.7		3.94	³ J _{5'6a'} = 2.4; ³ J _{5'6b'} = 7.2				
C-6	61.8	144.0		3.70; 3.94	³ J _{6a'6b'} = 12.6				
Glycerate									
C-1	177.6							3.0	
C-2	77.5	144.9	7.9	4.32	³ J _{2'3a} = 2.8; ³ J _{2'3b} = 7.3				
C-3	65.7	145.9	3.7	3.99; 3.89	³ J _{3a'3b'} = 11.0				
Phosphate P							5.0; 4.6	4.4 ^a	6.5

^a Value was determined at pH 8.0.^b pK_a values were determined based on the pH dependence of the chemical shift of the resonance due to H-C₂ of the glycerate moiety (3.0) and the pH dependence of the chemical shift of the phosphorus resonance (6.5).

TABLE II

Kinetic parameters for the substrates involved in the synthesis of mannosylglycerate in *P. horikoshii* and effect of Mg²⁺, Na⁺, and K⁺ ions

Parameters	Substrate	MPGS ^a	MPGP ^a
K _m (μM)	GDP-man	170	
	3-PGA	140	
	MPG		134
V _{max} (μmol/min·mg protein)		186	111
Mg ²⁺ ^b			
0 mM		46	58
15 mM		100	100
NaCl ^b			
0 mM		100	100
50 mM		89	100
150 mM		65	100
KCl ^b			
0 mM		100	100
50 mM		91	100
150 mM		82	100

^a All assays were carried out at 98 °C as described under "Experimental Procedures."^b Expressed as percentage of the maximum activity.FIG. 4. Temperature dependence of the recombinant MPG synthase (●) and the recombinant MPG phosphatase (○) of *P. horikoshii*. The enzyme activities were determined between 30 and 108 °C under the conditions described under "Experimental Procedures."

phorylated intermediate, mannosyl-3-phosphoglycerate, which in turn is dephosphorylated to α-mannosylglycerate (Fig. 7). Thus, the synthesis of MG can be traced from the key glycolytic metabolite glucose 6-phosphate as follows: glucose 6-phosphate is converted to fructose 6-phosphate by the action of a novel type of phosphoglucose isomerase (EC 5.3.1.9), recently identified in *P. furiosus* (27), which exhibits extensive sequence homology to *P. horikoshii* ORF *PH1956* (83% sequence identity). Fructose 6-phosphate can then be isomerized to mannose 6-phosphate by phosphomannose isomerase (*PH0925*), puta-

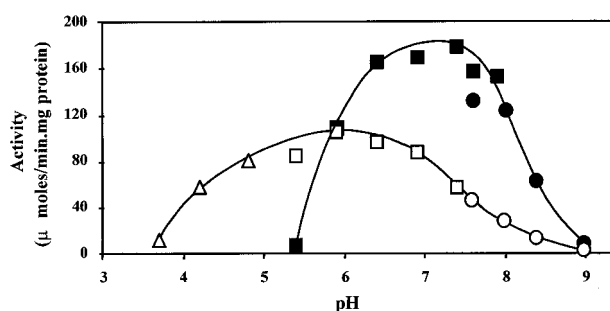
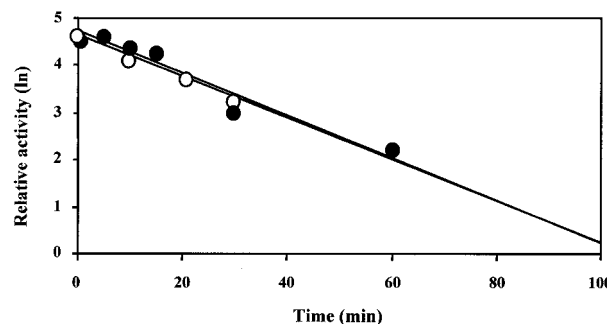
FIG. 5. pH dependence of the recombinant MPG synthase (solid symbols) and the recombinant MPG phosphatase (open symbols) of *P. horikoshii*. The enzyme activities were determined between pH 3.5 and 9.0 in acetate buffer (△), BisTris/propane (■ and □) and CAPSO (● and ○).

FIG. 6. Thermostability of recombinant MPG synthase (●) and MPG phosphatase (○). Enzymes were incubated at 98 °C. Samples were withdrawn and examined for activity at the same temperature. The half-life for thermal inactivation at 98 °C of MPG synthase and MPG phosphatase was 16 and 15.6 min, respectively.

tively identified as a bifunctional PMI/M1P-GT (28, 29). Mannose 6-phosphate is converted to mannose 1-phosphate by phosphomannose mutase (*PH0923*). The putative bifunctional enzyme encoded by *PH0925* could convert mannose 1-phosphate to GDP-mannose in the next step, using GDP as substrate. The assertion that *PH0925* encodes bifunctional PMI/M1P-GT is strongly supported by the presence of two conserved domains that are commonly found in type II PMI enzymes that possess both catalytic activities (29). The subsequent step involves the formation of MPG from GDP-mannose and 3-phosphoglycerate by MPG synthase (*PH0927*). MPG is finally converted to MG by MPG phosphatase (*PH0926*) (Fig. 7).

The proposed pathway in *P. horikoshii* is also supported by the genetic organization of *PH0927*, *PH0926*, *PH0925*, and

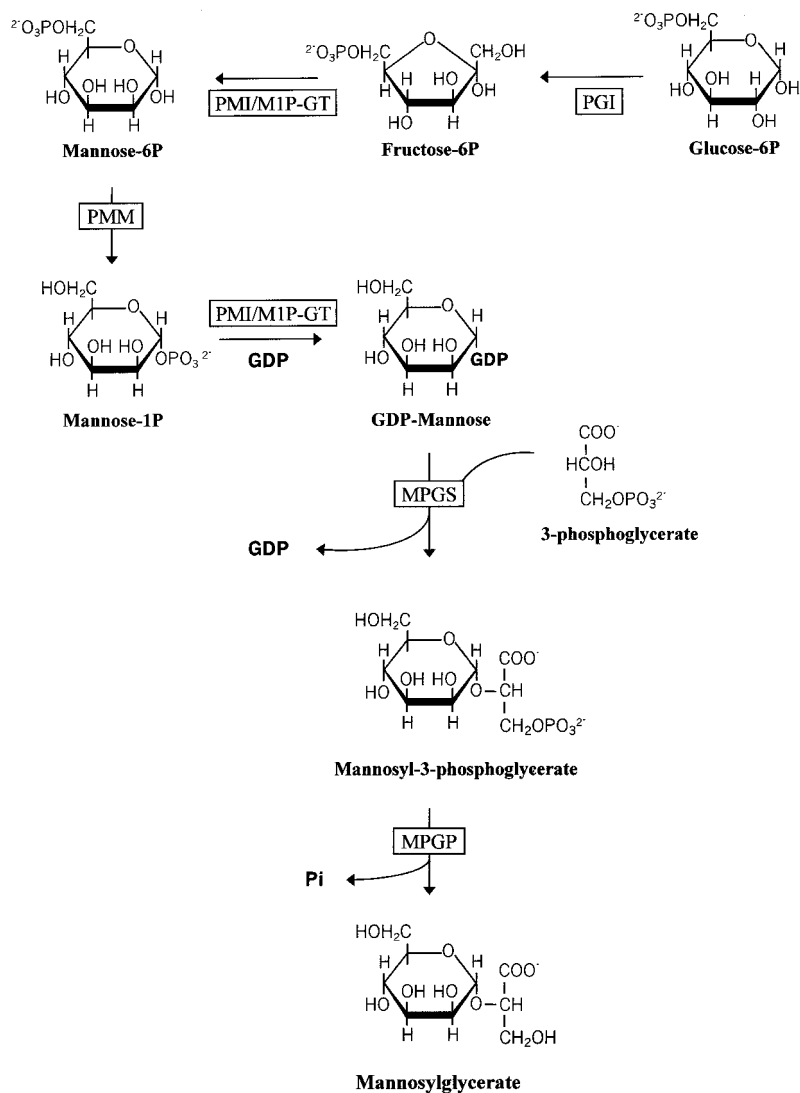


FIG. 7. Proposed pathway for the synthesis of mannosylglycerate in *P. horikoshii*. PGI, phosphoglucose isomerase (PH1956); PMI/M1P-GT (PH0925, NCBI accession number BAA30021); PMM, phosphomannose mutase (PH0923, NCBI accession number BAA30019); MPGS, mannosyl-3-phosphoglycerate synthase (PH0927, NCBI accession number BAA30023); MPGP, mannosyl-3-phosphoglycerate phosphatase (PH0926, NCBI accession number BAA30022).

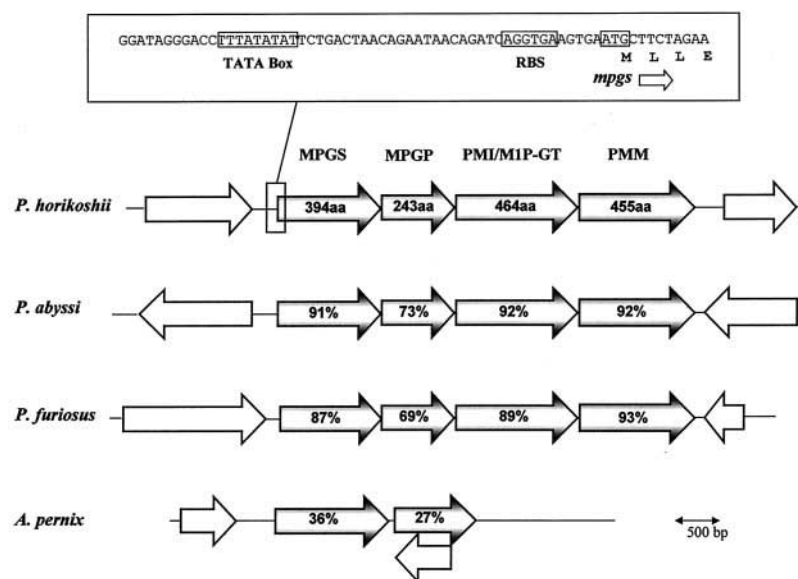


FIG. 8. Genomic organization and flanking regions of the MG operon in four archaeal species, *P. horikoshii*, *P. abyssi*, *P. furiosus*, and *A. pernix*. Arrows represent identified or putative genes (shaded) and unknown open reading frames (open) and their direction. The percentage of amino acid (aa) identity between homologous sequences is indicated inside the arrows. The enlargement shows the promoter region upstream *mpps* gene with the consensus TATA box, possible ribosome-binding site, and ATG start codon in boxes.

PH0923 (Fig. 8), because these four genes are organized in an operon-like structure. Moreover, the occurrence of a consensus archaeal AT-rich promoter sequence (TTTATATA) directly upstream of PH0927 indicates the formation of a polycystronic mRNA transcript (30, 31). It should be pointed out that other

candidate mannose-1-phosphate guanylyltransferase (e.g. PH1697 and PH1022) and phosphomannose mutase (e.g. PH1210) genes are found in *P. horikoshii* that may be involved in the synthesis of mannose derivatives needed for other biosynthetic purposes, such as mannose-containing polysaccha-

rides. Therefore, we hypothesize that genes *PH0923* and *PH0925* encoding phosphomannose mutase and bifunctional phosphomannose isomerase/mannose-1-phosphate guanylyltransferase, may be committed to MG synthesis, being expressed upon osmotic sensing.

Unlike *R. marinus*, where two pathways for the synthesis of MG have been identified (15), only the two-step pathway, proceeding through a phosphorylated intermediate, has been detected in the archaeon *P. horikoshii*. An enzyme activity for the direct conversion of GDP-mannose and D-glycerate to MG by cell extracts was not detected in this organism. A search of the *P. horikoshii* genome revealed an ORF (*PH1879*) that had 29% sequence similarity with the *R. marinus* MG synthase. However, the recombinant gene product had no measurable activity for the synthesis of MG.² The pathway for the synthesis of MG in *P. horikoshii* appears to be similar to those described for the synthesis of osmolytes like trehalose (32), glucosylglycerol (33), and galactosylglycerol (34), insofar as all proceed via two-step pathways involving a phosphorylated intermediate. The existence of a single pathway for the synthesis of MG in *P. horikoshii* instead of the branched pathway of *R. marinus* probably imposes a lower flexibility on the regulation of MG synthesis in response to osmotic and/or thermal stress. However, the significance of the presence of two pathways in *R. marinus*, as well as the accumulation of the compatible solute mannosylglyceramide, which is unique to this organism, remains elusive.

A search of data bases revealed that genes encoding MPG synthase (*PH0927*) and MPG phosphatase (*PH0926*) have homologues, with sequence identities of at least 87 and 69%, respectively, in *Pyrococcus abyssi* and *P. furiosus* genomes (National Centre for Sequencing, France (www.genoscope.cns.fr) and Utah Genome Center website (www.genome.utah.edu)). Moreover, the corresponding genes in these *Pyrococcus* spp. are also organized in operon-like structures with four genes that include putative phosphomannose mutase and bifunctional phosphomannose isomerase/mannose-1-phosphate guanylyltransferase. Lower sequence homology (37 and 27% identity) to the MPG synthase and MPG phosphatase genes of *Pyrococcus* spp. were also detected in the crenarchaeote *A. pernix* genome (37), but putative phosphomannose mutase and bifunctional phosphomannose isomerase/mannose-1-phosphate guanylyltransferase were not found immediately downstream from MPG synthase/MPG phosphatase genes (Fig. 8).

The MPG synthase sequence contains one highly conserved motif, DXD, found in several families of glycosyltransferases that use nucleoside diphosphate sugars as substrates (38). Glycosyltransferases are classified as “inverting” or “retaining” enzymes according to whether their catalytic mechanisms result in inversion or retention of the anomeric configuration of substrates (Carbohydrate-Active enzymes (afmb.cnrs-mrs.fr/~pedro/CAZY) and see Refs. 39, 41, and 42). A PSI-BLAST search with the MPG synthase sequence revealed a conserved domain specific of family 2 glycosyltransferases (Fig. 3), which are “inverting” enzymes. However, our data show that MPG synthase retains the α -configuration of the substrate GDP-mannose and therefore cannot be classified as a family 2 glycosyltransferase. The same reasoning is applicable to the MG synthase characterized earlier in *R. marinus* (15). MPG synthase from *P. horikoshii* does not share significant amino acid sequence homology with other known sugar transferases nor with MG synthase from *R. marinus* (15). In view of the apparent peculiarity of the amino acid sequences of these two GDP-mannose-dependent enzymes, and the retaining character of

their catalytic mechanism, the elucidation of their three-dimensional structures would be most important to reconcile these findings.

The sequence of *P. horikoshii* MPG phosphatase contained a conserved domain found in trehalose-6-phosphate phosphatases (Fig. 2); however, trehalose 6-phosphate was not a substrate for MPG phosphatase. Interestingly, MPG phosphatase has no significant sequence homology with other known phosphatases, and this divergence may explain the very high specificity of this enzyme for MPG. The glucosylglycerol-phosphate phosphatase from *Synechocystis* spp. is another example of a phosphatase that possesses a consensus motif common to acid phosphatases (43), very weak sequence similarity with other sugar phosphatases, and a very high specificity for its substrate (44).

MPG synthase and MPG phosphatase had maximum activity at very high temperatures but no detectable activity at low temperatures, as might be expected from results with other intracellular enzymes from hyperthermophilic organisms (35, 45). More interesting is the low thermostability of both enzymes at 98 °C, the optimum temperature for growth of the organism. The weak intrinsic thermostability of MPG synthase and MPG phosphatase, from an organism that can grow at temperatures close to the boiling point of water, implies the existence of extrinsic stabilizing factors and/or high turnover rates to ensure the functionality of these enzymes *in vivo*. It should be noted that other intracellular enzymes from hyperthermophiles also display various degrees of thermostability some of which are also surprisingly low (36).

In addition to mannosylglycerate *P. horikoshii* accumulated di-*myo*-inositol phosphate and small amounts of trehalose. It is interesting to note that genes for the synthesis of trehalose could not be identified in the genome of this organism, nor could we find the corresponding activities in cell extracts. Therefore, the accumulation of this disaccharide is exclusively due to uptake from the yeast extract in the growth medium, through a high affinity ABC maltose/trehalose transporter recently identified in *Thermococcus litoralis* (40).

The pathway for the synthesis of mannosylglycerate in the hyperthermophilic archaeon *P. horikoshii* proposed by us, as well as the characterization of the enzymes, genes, and operon-like structure, represents an essential step toward the elucidation of osmo/thermoregulation through the accumulation of compatible solutes in thermophilic and hyperthermophilic organisms.

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