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SHORT COMMUNICATION

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# Glucose-6-phosphate isomerase from the hyperthermophilic archaeon *Methanococcus jannaschii:* characterization of the first archaeal member of the phosphoglucose isomerase superfamily

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**Abstract** ORF MJ1605, previously annotated as *pgi* and coding for the putative glucose-6-phosphate isomerase (phosphoglucose isomerase, PGI) of the hyperthermophilic archaeon *Methanococcus jannaschii*, was cloned and functionally expressed in *Escherichia coli*. The purified 80-kDa protein consisted of a single subunit of 45 kDa, indicating a homodimeric ($\alpha_2$) structure. The $K_m$ values for fructose 6-phosphate and glucose 6-phosphate were 0.04 mM and 1 mM, the corresponding $V_{max}$ values were 20 U/mg and 9 U/mg, respectively (at 50 °C). The enzyme had a temperature optimum at 89 °C and showed significant thermostability up to 95 °C. The enzyme was inhibited by 6-phosphogluconate and erythrose-4-phosphate. RT-PCR experiments demonstrated in vivo expression of ORF MJ1618 during lithoautotrophic growth of *M. jannaschii* on $H_2/CO_2$. Phylogenetic analyses indicated that *M. jannaschii* PGI was obtained from bacteria, presumably from the hyperthermophile *Thermotoga maritima*.

**Keywords** Phosphoglucose isomerase · hyperthermophilic archaea · *Methanococcus jannaschii* · Phosphoglucose isomerase superfamily

## Introduction

Glucose-6-phosphate isomerase or phosphoglucose isomerase (PGI; EC 5.3.1.9) catalyzes the reversible isomerization of glucose 6-phosphate to fructose 6-phosphate. PGI plays a central role in sugar metabolism of eukarya, bacteria, and archaea, both in glycolysis via the Embden-Meyerhof pathway in eukarya and bacteria and in the modified versions found in archaea as well as in gluconeogenesis where the enzyme operates in the reverse direction (for literature, see Hansen et al. 2001).

PGIs from the domains of eukarya and bacteria are well-studied enzymes (e.g. Charles and Lee 1980; Hesman et al. 1991; Nyame et al. 1994; Schreyer and Bock 1980; Sun et al. 1990). Crystal structures have been determined for the eukaryotic PGIs from human, pig, rabbit, and the bacterium *Bacilllus stearothermophilus* (for literature, see Arsenieva et al. 2002; Davies et al. 2003; Lee et al. 2001). Sequence comparison of PGIs ranging from bacteria to mammals have revealed two conserved signature patterns of the PGI superfamily (Falquet et al. 2002).

So far, little information is available on PGIs from the archaeal domain. Only two archaeal PGIs have been been characterized, from the hyperthermophilic euryarchaeon *Pyrococcus furiosus* (Hansen et al. 2001; Verhees et al. 2001) and from closely related *Thermococcus litoralis* (Jeong et al. 2003). Both PGIs belong to the cupin superfamily (Dunwell et al. 2001) and represent a convergent branch of PGI evolution. Cupin-type PGIs are also present in the euryarchaea *Archaeoglobus fulgidus* and *Methanosarcina mazei* (Hansen and Schönheit, unpublished). Although more than 300 bacterial and eukaryal PGI sequences from the PGI superfamily are known (see http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00342) (Bateman et al. 2001), only two homologs of this superfamily have been identified in available archaeal genomes: ORF MJ1605 in the hyperthermophilic methanogen *Methanococcus jannaschii* (Bult et al. 1996) and ORF VNG1992G in the extreme halophile (Ng et al. 2000); however, their coding function in the metabolism of these archaea has not been proven. Here we report the functional expression of ORF MJ1605 and its characterization as an extremely thermophilic PGI of the PGI-superfamily.

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## Materials and methods

### Cloning of ORF MJ1605 and RT-PCR

ORF MJ1605 was cloned and functionally overexpressed in *Escherichia coli* as follows: The coding region of ORF MJ1605, anno-

tated as putative *pgi* (Bult et al. 1996), was amplified by PCR with *Pwo* polymerase (PEQLAB, Germany) from the genomic DNA of *M. jannaschii*. Using the primers 5′GAAATATGCTAACATATGCTAAGTTATGATTAC3′ (forward) and 5′GAAAAAGAACTCGAGATATTATTTATTGTTTAATC3′ (reverse), restriction sites (underlined) for *NdeI* and *XhoI*, respectively, were introduced. Following amplification and *NdeI*/*XhoI* double-digestion, the PCR product was inserted by T4 DNA ligase (Roche Diagnostics) into a pET-17b (Novagen) expression vector linearized by *NdeI*/*XhoI* double-digestion. The resulting plasmid, pET-14b-*pgi*, was introduced into *E. coli* JM109 and BL21-CodonPlus(DE3)-RIL (Stratagene) via transformation. The gene sequence was confirmed by standard methods (Sanger et al. 1977). Exponentially grown cells of *M. jannaschii* (100 mg) were disrupted by freezing in liquid nitrogen and subsequent thawing. RNA was isolated using the RNeasy isolation kit (Qiagen) as specified by the manufacturer. RT-PCR was carried out by the Qiagen oneStep RT-PCR kit using the PCR primers. RNA that has been not incubated with reverse transcriptase was used as negative control.

### Functional overexpression of *pgi* in *E. coli* and purification of recombinant *M. jannaschii* PGI

Transformed *E. coli* BL21-CodonPlus(DE3)-RIL cells were grown at 37 °C to an optical density of 0.8 at 600 nm, and *pgi* expression was initiated by induction with 0.4 mM IPTG. After 4 h of further growth ($OD_{600}$~3.2), the cells were harvested by centrifugation at 4 °C. Cell extracts were prepared by French press treatment ($1.3\times10^8$ Pa) of cell suspensions in buffer A (100 mM Tris/HCl, 50 mM NaCl, 2 mM EDTA, 1 mM DTE, pH 8.0, 25 °C]). After ultracentrifugation (100,000×*g* for 60 min) the supernatant was heated for 30 min at 75 °C and centrifuged again (10,000×*g* for 30 min). All chromatographic steps were carried out at 4 °C. The solution was adjusted to pH 6.2 and applied onto a DEAE-Sepharose column (22×2.2 cm) previously equilibrated with buffer B (50 mM piperzine, pH 6.2). After washing the column with 70 ml buffer B and 70 ml buffer C (50 mM piperzine, pH 5.3), protein was eluted with two NaCl gradients, from 0–0.2 M (60 ml) and 0.2–2M (60 ml). PGI-containing fractions were concentrated by ultrafiltration (exclusion size 20 kDa) and applied to a Superdex 200 gel filtration column (1.6×60 cm) equilibrated with buffer D (50 mM Tris-HCl, 150 mM NaCl, pH 7.5). PGI eluted from this step was pure.

### Enzyme assays

PGI activity (G6P ⇌F6P ) in both directions was determined using either a discontinuous assay at 50–96 °C (at least six parallel assays stopped at different time intervals) or a continuous assay at 20–50°C, as previously described (Hansen et al. 2001), except for the standard assay mixture, which contained 100 mM Tris-HCl, pH 6.3 (50 °C), 0.5 mM $NADP^+$, 10 mM fructose 6-phosphate, 1.4 U glucose-6-phosphate dehydrogenase, 1 µg PGI (glucose 6-phosphate-formation); 100 mM Tris-HCl, pH 6.3 (50 °C), 10 mM glucose 6-phosphate, 2 mM ATP, 5 mM $MgCl_2$, 0.6 mM NADH, 0.05 U (50 °C) *Thermotoga maritima* ATP-dependent 6-phosphofructokinase (ATP-PFK) (Hansen et al. 2002), 0.9 U fructose-1,6-bisphosphate (F-1,6BP) aldolase, 5 U triose phosphate isomerase (TIM), 1.5 U glycerol-3-phosphate dehydrogenase (fructose 6-phosphate formation), 1 µg PGI. The pH dependence of the enzymes was measured between 3.8 and 8.7 at 50 °C using either acetate (pH 3.8–5.8), piperazine (pH 5.0–6.5), or Tris-HCl (pH 7.0–8.2), ethanolamine (pH 8.0–8.7). The inhibitory effects of erythrose 4-phosphate and 6-phosphogluconate were studied at 50 °C. As an alternate substrates, mannose-6-phosphate (M6P) was used instead of glucose 6-phosphate. Glucose was assayed as previously described (Hansen et al. 2001) in order to detect potential PGI-catalyzed frucose/glucose isomerization.

### Temperature dependence and thermal stability

The temperature dependence of PGI activity was measured between 20 and 96 °C in 50 mM sodium phosphate buffer, pH 6.3 (which is not inhibitory at that concentrations), using standard concentrations of glucose 6-phosphate and $NADP^+$ which ensured specific activities close to $V_{max}$. The thermostability of the purified enzyme (10 µg in 40 µl 200 mM Tris-HCl, pH 6.3) was tested in sealed vials incubated at temperatures between 80 and 100 °C for 5–120 min. The vials were then cooled on ice for 10 min and residual enzyme activity was tested at 50 °C in the continuous assay and compared to controls (unheated samples).

### Sequence handling

Sequence alignments were constructed with the neighbor-joining method of ClustalX (Thompson et al. 1997) using the xy GONNET matrix. Phylogenetic trees were constructed using the neighbor-joining (NJ) option of ClustalX 1.8 and the maximum likelihood phylogenetic analysis of TREE-PUZZLE v.5 (QP) carried out by quartet-puzzling (Schmidt et al. 2002). Confidence limits were estimated by 100 bootstrapping replicates. PGI sequencs were retrieved from the pfam-database (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00342), a putative PGI from *Haloarcula marismortui* from (http://zdan2.umbi.umd.edu).

## Results and discussion

### Purification and molecular properties

ORF MJ1605 contains 1,203 bp coding for a polypeptide of 401 amino acids with a calculated molecular mass of 45.674 kDa. The coding function of MJ1605 was proved by its functional overexpression in *E. coli*. Recombinant MjPGI was purified approximately seven-fold from *E. coli* and about 33 mg protein was obtained from ~ 0.5 g soluble protein of transformed *E. coli* cells. Purified MjPGI had a native molecular mass of about 80 kDa, as determined by gel filtration on Superdex 200, and was composed of a single 45-kDa subunit, indicating a homodimeric structure. A homodimeric structure is a typical property of most characterized PGIs of the PGI superfamily from eubacteria and eukarya (see, e.g. Schreyer and Bock 1980, Ruijter and Visser 1999). Exceptions are the PGIs from two thermophilic *Bacillus* species, *B. stearothermophilus* and *B. caldotenax*, which have been shown to be homotetrameric enzymes (Muramatsu and Nosoh 1971; Takama and Nosoh 1980). With a subunit size of 45 kDa, MjPGI is the smallest PGI of the PGI superfamily and thus might represent the minimal core structure of a PGI of this family.

### Catalytic properties

The rate dependence of the enzyme on fructose 6-phosphate and glucose 6-phosphate followed Michaelis-Menten kinetics, with $K_m$ values of 0.04 and 1 mM; the corresponding $V_{max}$ values were 21 and 9 U/mg (50 °C), respectively. The pH optimum of PGI with fructose 6-phosphatewas at 6.3; 50% remaining activity was observed at pH 5.3 and pH 7. PGI from *M. jannaschii* did not show activity with

mannose-6-phosphate and with the non-phosphorylated hexoses glucose and fructose. MjPGI was inhibited by 6-phosphogluconate and erythrose 4-phosphate, typical inhibitors of the PGI superfamily. Inhibition of PGI by both compounds was competitive, e g. addition of 2.5 µM erythrose 4-phosphateresulted in a 42-fold increase of the $K_m$ for fructose 6-phosphate (from 0.04 to 1.8 mM), without affecting the $V_{max}$ values (at 50 °C); the respective $K_i$ values were 20 and 60 µM. These values are in the same order of magnitude as those of other PGIs of the superfamily (Pradhan and Nadkarni 1980; Takama and Nosoh 1980; Schreyer and Bock 1980). This suggests that the mechanisms of substrate binding and of isomerization of the archaeal PGI are very similar to those of characterized eukaryal and bacterial PGIs.

## Temperature dependence and thermostability

PGI from *M. jannaschii* showed a temperature optimum at 89 °C (Fig. 1A), which is by far the highest value of all PGIs of the superfamily characterized so far. An activation energy of 40 kJmol$^{-1}$ was calculated from the linear part of the Arrhenius plot between 23 and 89 °C. Furthermore, the enzyme exerted a high thermostability (Fig. 1B): It did not lose activity upon incubation at 80 C for about 120 min and still had a half-life at 95 °C of 40 min. At 100 °C, an almost complete loss of activity was observed after 30 min. In contrast, PGI from the thermophilic *Bacillus* strains *B. staerothermophilus* and *B. caldotenax* had temperature optima of 70 and 77 °C, respectively; both PGIs were inactivated about 50% upon incubation for 2 h at about 65 °C (Takama and Nosoh 1980; Muramatsu and Nosoh 1971). The extremely high temperature optimum of activity and thermostability of *M. jannaschii* PGI is in accordance with its physiological function under hyperthermophilic growth conditions.

## In vivo transcription of *pgi* and PGI activity in extracts of *M. jannaschii*

To test whether *pgi* is transcribed in vivo, RT-PCR experiments were carried out to detect mRNA formation. Total RNA was extracted from *M. jannaschii* cells grown lithoautotrophically on $H_2$ and $CO_2$ as energy and carbon source. *pgi*-specific RNA was amplified as cDNA by RT-PCR, and a *pgi*-specific cDNA of the expected length (1,243 bp) was detected, indicating in vivo transcription of *pgi* during lithoautotrophic growth. Extracts of lithoautotrophic grown *M. jannaschii* cells contain PGI activity of 2–5 mU/mg (50 °C). Fractionation of the cell extract by gel-filtration chromatography on Superdex 200 revealed that PGI activity elutes in one peak at an elution volume identical to that obtained for the recombinant enzyme, which has an apparent molecular mass of about 80 kDa. This indicates that the physiologically active PGI in *M. jannaschii* is also an 80-kDa protein, probably identical to the ORF MJ1605 gene product. Since the apparent molecular masses of the archaeal cupin-type PGIs found in *Pyrococcus* and *Thermococcus* are much smaller (about 45 kDa) (Hansen et al. 2001; Verhees et al. 2001; Jeong et al. 2003), the presence of this PGI type can most likely be excluded.

## Sequence comparison and phylogenetic analysis

The MjPGI shows a significant degree of similarity (27–49%) to PGIs of the PGI superfamily thus defining this archaeal enzyme as a member of the PGI superfamily, with putative homologs from *T. maritima* (Q9X1A5, 49%) and *Haloarcula marismortui* (http://zdan2.umbi.umd.edu, 47%) being the most similar. The MjPGI sequence deviates from both PGI consensus patterns by two residues (not shown). The putative PGIs from *H. marismortui*, *Halo-*

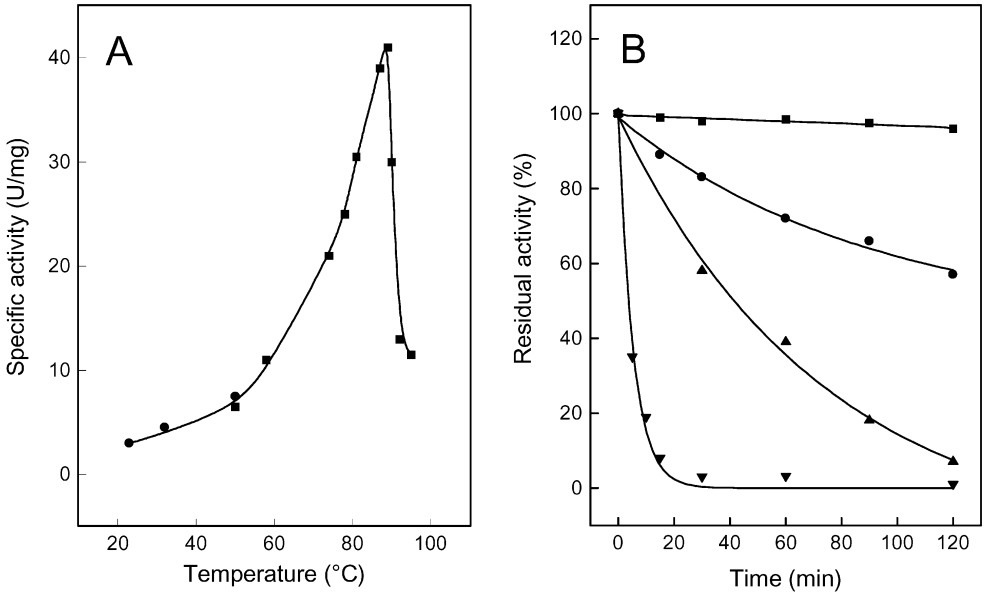


**Fig. 1** **A** Effect of temperature on the specific activity of the *Methanococcus jannaschii* phosphoglucose isomerase (PGI). Enzyme activity was measured in the direction of glucose 6-phosphate formation. ● Continuous assay, ■ discontinuous assay. The assay mixture contained 1.4 µg enzyme. **B** Thermostability of *M. jannaschii* PGI. Ten µg enzyme were incubated at 80 °C ■, 90°C ●, 95°C ▲. and 100°C ▼. At the times indicated, aliquots were withdrawn and assayed for residual activity at 50 °C in the direction of glucose 6-phosphate formation; 100% activity corresponded to a specific activity of PGI of 9 U/mg

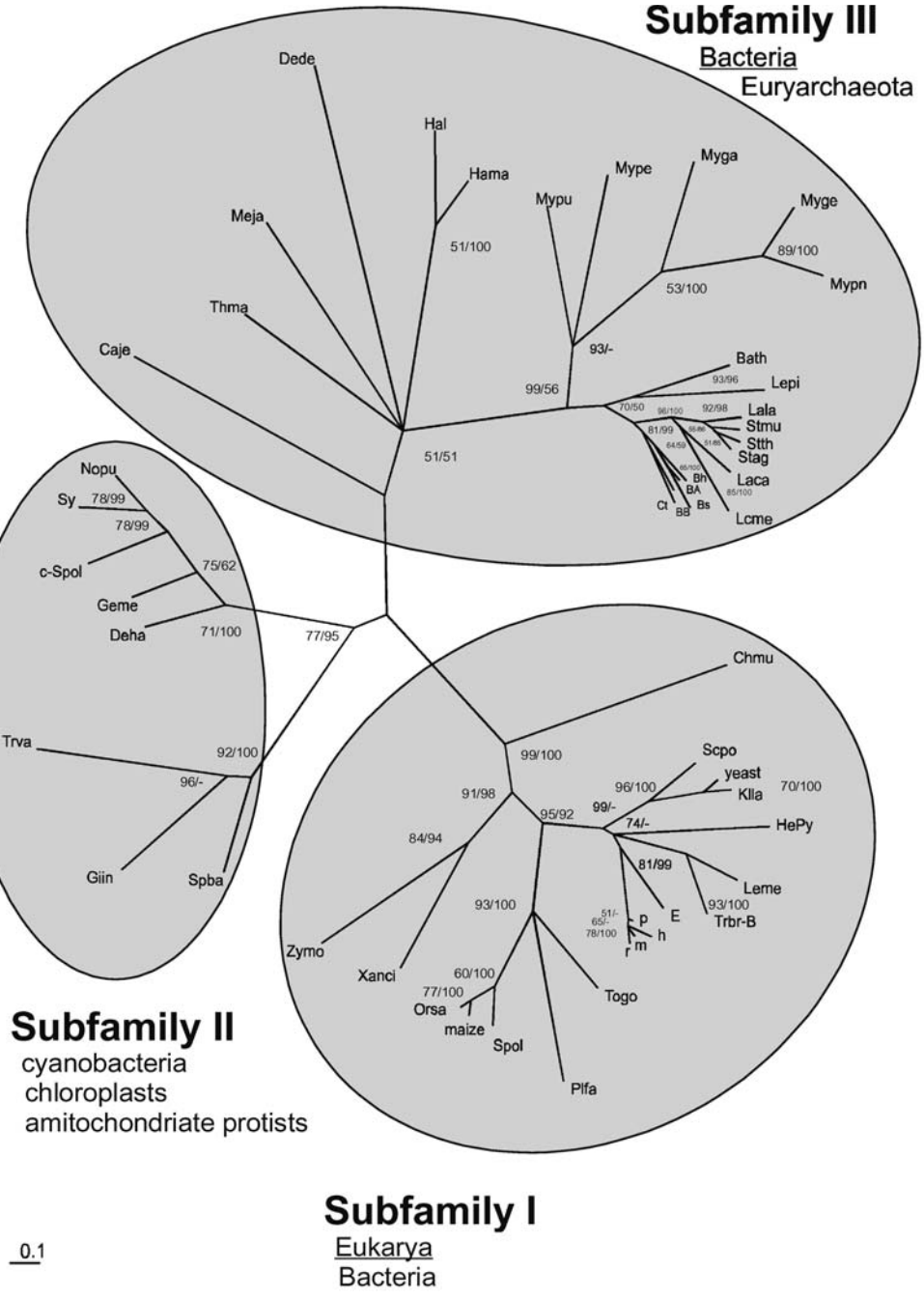


**Fig. 2** Phylogenetic tree of selected PGIs from the PGI superfamily from bacteria, eukarya, and archaea. The *numbers* at the nodes are bootstrapping values according to neighbor-joining (first value) and quartet puzzling (second value). NCBI accession numbers of SwissProt identifiers: Meja *Methanococcus jannaschii* Q59000; Hama *Haloarcula marismortui* (http://zdan2.umbi.umd.edu); Hal *Halobacterium* NRC1 Q9HNQ6; Dede *Desulfovibrio desulfuricans* (G20) 23473348; Thma *Thermotoga maritima* Q9X1A5; Mypn *Mycoplasma pneumoniae* P78033; Myge *Mycoplasma genitalium* P47357; Myga *Mycoplasma gallisepticum* Q9KX58; Mype *Mycoplasma penetrans* Q8EVU1; Lepi *Leptospira interogans* Q8EZG6; Bath *Bacteroides thetaiotaomicron* Q8A5W2; Stag *Streptococcus agalactiae* Q8E6X1; Stth *Streptococcus thermophilus* AAL35379; Stmu *Streptococcus mutans* Q9X670; Lala *Lactococcus lactis* P81181; Laca *Lactobacillus casei* Q9WXF6; Lcme *Leuconostoc mesenteroides* 23024190; Bs *Bacillus subtilis* P80860; BA *Bacillus stearothermophilus* A P13375; Bh *Bacillus halodurans* Q9K7L8; Ct *Clostridium thermocellum* 23021679; BB *Bacillus stearothermophilus* B P13376; Mypu *Mycoplasma pulmonis*; Caje *Campylobacter jejuni* Q9PMD4; Zymo *Zymomonas mobilis* P28718; Xaci *Xanthomonas campestri* O68824; Orsa *Oryza sativa* P42862; maize zea mays P49105; Spol *Spinacia oleracea* CCAA03983; Togo *Toxoplasma gondii* Q9XY88; Plfa *Plasmodium falciparum* P18240; Trbr-B *Trypanosoma brucei* B P13377; Leme *Leishmania mexicana* P42861; Klla *Kluyveromyces lactis* P12341; yeast P12709; Scpo *Schizosaccharomyces pombe* P78917; human P06744; p pig P08059; r rabbit Q9N1E2; m mouse P06745; E *Escherichia coli* P11537, Hepy *Helicobacter pylori* O25781; Chmu *Chlamidia muridarum* AE002334; Giin *Giardia intestinalis* AAK49040; Trva *Trichomonas vaginalis* AAL56573; Nopu *Nostoc punctiforme* 23125809; Sy *Synechocystis* sp. P52983, c-Spol *Spinacia oleracea* chloroplast T09153; Geme *Geobacter metallireducens* 23053592; Deha *Desulfitobacterium hafniense* 23114897

*bacterium* NRC1 exhibit five and seven deviations, respectively. Due to the high homology to MjPGI, we expect these halobacterial ORFs to encode PGIs as well. All conserved residues, which are crucial for substrate recognition/binding or catalysis, as deduced from various crystal structures or mutagenesis studies (Lee et al. 2001; Meng et al. 2001), are conserved in these euryarchaeal PGIs sequences as well. However, a glycine, conserved in all other PGIs sequences, is substituted by a proline (Pro-174 MjPGI) in these euryarchaeal PGI sequences, which might be due to phylogenetic influences. Gly-271 of human PGI is part of a GlyGlyArg loop structure that is conserved in all annotated bacterial and eukaryotic PGIs. The absence of side chains of the Gly-271 and Gly-272 has been proposed to permit binding of the substrate (Davies et al. 2003; Lee et al. 2001), which is apparently also possible with the ProGlyArg structure found in the archaeal PGIs.

MjPGI is the first characterized archaeal member of the PGI superfamily, thus allowing phylogenetic studies. A phylogram including both characterized and putative PGIs from the PGI superfamily from eukarya, bacteria, and archaea is given in Fig. 2. In accordance with previous phylogenetic studies (Henze et al. 2001), the PGIs cluster in at least three groups. Several bacteria (*Escherichia coli, Helicobacter pylori, Chlamydia muridarum, Xanthomonas campestri*), which are symbionts or parasites of eukarya, cluster within the eukaryal Subfamily I sequences. This might be explained by independent lateral gene transfers from eukarya as proposed for the *E. coli* PGI (Katz 1996). The monophylogenetic origin of the archaeal PGIs is not supported by phylogenetic analysis. The euryarchaeal sequences from *M. jannaschii, Halobacterium* NRC1, and *H. marismortui* are rather included in subfamily III, which also comprises PGIs from various bacteria. The euryarchaeal sequences fall into a subgroup with the putative bacterial PGIs from *T. maritima* and *Desulfovibrio desulfuricans* G20, which might even be interpreted as a separate subfamily. However, the basal node(s) of this cluster cannot be accurately resolved, neither by maximum-likelihood-based quartet-puzzling analysis nor by neighbor-joining-analysis, but might be improved when more related PGI sequences become available. Within this subgroup, MjPGI clusters closest with the putative *T. maritima* PGI, which might indicate a lateral gene transfer of *pgi* between a hyperthermophilic *Thermotoga* species to the hyperthermophilic *M. jannaschii*, a phenomenon that has been proposed to occur at high frequency between *T. maritima* and hyperthermophilic archaea (Nelson et al. 1999).

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