Identification and Characterization of an Archaeal Kojibiose Catabolic Pathway in the Hyperthermophilic *Pyrococcus* sp. Strain ST04

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A unique gene cluster responsible for kojibiose utilization was identified in the genome of *Pyrococcus* sp. strain ST04. The proteins it encodes hydrolyze kojibiose, a disaccharide product of glucose caramelization, and form glucose-6-phosphate (G6P) in two steps. Heterologous expression of the kojibiose-related enzymes in *Escherichia coli* revealed that two genes, Py04_1502 and Py04_1503, encode kojibiose phosphorylase (designated PsKP, for *Pyrococcus* sp. strain ST04 kojibiose phosphorylase) and β -phosphoglucosylmutase (PsPGM), respectively. Enzymatic assays show that PsKP hydrolyzes kojibiose to glucose and β -glucose-1-phosphate (β -G1P). The K_m values for kojibiose and phosphate were determined to be 2.53 ± 0.21 mM and 1.34 ± 0.04 mM, respectively. PsPGM then converts β -G1P into G6P in the presence of 6 mM $MgCl_2$. Conversion activity from β -G1P to G6P was 46.81 ± 3.66 U/mg, and reverse conversion activity from G6P to β -G1P was 3.51 ± 0.13 U/mg. The proteins are highly thermostable, with optimal temperatures of 90°C for PsKP and 95°C for PsPGM. These results indicate that *Pyrococcus* sp. strain ST04 converts kojibiose into G6P, a substrate of the glycolytic pathway. This is the first report of a disaccharide utilization pathway via phosphorolysis in hyperthermophilic archaea.

Heterotrophs possess a myriad of mechanisms to assimilate carbohydrates from the environment. They are commonly composed of extracellular hydrolases, transporter complexes, and regulatory systems (1, 2). Among these, maltose and maltodextrin uptake systems have been widely investigated (3, 4). In Gram-negative bacteria, such as *Escherichia coli*, maltose is assimilated through an ATP-binding cassette (ABC) transporter system and intracellular amylases, including 4- α -glucanotransferase, maltodextrin glucosidase, and maltodextrin phosphorylase (5, 6). However, the Gram-positive bacterium *Bacillus subtilis* takes up maltose and maltodextrin using a phosphoenolpyruvate-dependent phosphotransferase system (PTS) mediated by a maltose-specific enzyme, IICB, and an ABC transporter containing a maltodextrin-binding protein (7, 8). Within *B. subtilis*, maltose is hydrolyzed into glucose and glucose-6-phosphate (G6P) by an NAD(H)-dependent 6-phospho- α -glucosidase. Alternatively, in *Lactococcus lactis* and *Lactobacillus sanfranciscensis*, maltose uptake occurs by an ATP-dependent permease system and is hydrolyzed by maltose phosphorylase, resulting in the production of glucose and β -glucose-1-phosphate (β -G1P) (9, 10). β -G1P is then converted into G6P by phosphoglucosylmutase and utilized as a substrate for glycolysis. A similar mechanism is found in *Clostridium phytofermentans*, which transforms nigerose to glucose and G6P using nigerose phosphorylase and β -phosphoglucosylmutase (1).

Maltose phosphorylase and nigerose phosphorylase are classified in glycoside hydrolase family 65 (GH65), along with kojibiose phosphorylase and trehalose phosphorylase. These disaccharide phosphorylases (DPases) are a distinct group of carbohydrate-active enzymes that break glycosyl linkages in a disaccharide with the use of inorganic phosphate (11). This hydrolysis, or phosphorolysis, reaction results in a glucosyl-phosphate and a glucose. Incubation of products leads to the reformation of disaccharides, since the reaction performed by DPases is reversible (12, 13). Although both inverting and retaining reactions are possible, depending on the anomeric configuration of the donor substrate, the majority of DPases are inverting enzymes (11). DPases also func-

tion as both glycoside hydrolases (GH) and glycosyl transferases (GT) due to their phosphorolysis and synthesis activities (11). While some DPases are classified as transferases, the phosphorolysis reaction *in vivo* is favored over the synthesis reaction, resulting in the production of glucosyl-phosphate that enters the glycolytic pathway without activation by a kinase (1, 14).

Carbohydrate degradation in the hyperthermophilic archaea *Pyrococcus* and *Thermococcus* typically proceeds via amylases and amylopullulanases together with maltose and maltodextrin transporter systems (4, 15). They catabolize glucose into pyruvate by a unique Embden-Meyerhof pathway that uses ADP-dependent glucokinase and phosphofructokinase and a ferredoxin-dependent glyceraldehyde-3-phosphate oxidoreductase (16, 17). In this study, we report on a novel kojibiose catabolic gene cluster in *Pyrococcus* sp. strain ST04 that encodes DPase and β -phosphoglucosylmutase. These proteins are responsible for the hydrolysis of kojibiose, a disaccharide produced by glucose caramelization, to glucose and β -G1P and the transformation of β -G1P to G6P, respectively. The resulting G6P might be used as a substrate in glycolysis. This study is the first report of phosphorolysis of kojibiose in archaea.

MATERIALS AND METHODS

Microbial strains and chemicals. Restriction endonuclease and *Pfu*-Ultra polymerase were purchased from New England BioLabs (Beverly, MA, USA) and Stratagene (La Jolla, CA, USA), respectively. Disaccharides used for the determination of enzyme activity were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or Wako Pure Chemical (Osaka,

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Japan). The medium for cultivation of *Pyrococcus* sp. strain ST04 were prepared as described by Osowski et al. (18). *E. coli* DH10B [F^- *araD139* Δ (*ara leu*)7697 Δ *lacX74 galU galK rpsL deoR* ϕ 80*lacZ* Δ M15 *endA1 nupG recA1 mcrA* Δ (*mrr hsdRMS mcrBC*)] was used as a host for DNA cloning, and *E. coli* BL21 CodonPlus(DE3)-RP [*E. coli* B F^- *ompT hsdS*($r_B^- m_B^-$) *dcm*⁺ Tet^r gal λ (DE3) *endA* Hte (*argU proL* Cam^r)] was used for expression studies. These strains were grown in Luria broth (LB) containing 1% (wt/vol) Bacto-tryptone, 0.5% (wt/vol) yeast extract, 0.5% (wt/vol) NaCl, and 100 μ g/ml ampicillin. The pGEM T-easy vector (Promega, Madison, WI, USA) and the pHCXHD vector, which was derived from the vector pHCEII-NdeI (BioLeaders Co., Daejeon, South Korea) (19), were used for PCR cloning and expression, respectively.

Construction of PsKP and PsPGM gene expression vectors. The genes for PsKP and PsPGM in *Pyrococcus* sp. strain ST04 were amplified by PCR using two pairs of primers whose designs were based on the whole genome sequence of *Pyrococcus* sp. strain ST04 (20). For cloning of the PsKP gene, primers 1502_EcoRV (5'-GAT ATC ATA TGG AGA TCA CCG TTG AAT ATA TTG G-3') and 1502_XhoI (5'-CTC GAG GGG TAT TAA CTT TGA CC-3') were designed to introduce EcoRV and XhoI recognition sites (underlined) into the product. Likewise, 1503_NdeI (5'-CAT ATG ATT GGA ATT ATT TGG GAT TT-3') and 1503_XhoI (5'-CTC GAG ACG GTG ATC TCC ATC CCC A-3') were used to amplify the PsPGM gene. The standard conditions for PCR were as follows: one cycle of denaturation at 94°C for 5 min, 20 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 3 min for the PsKP gene and for 1 min for the PsPGM gene, and extra extension at 72°C for 8 min. The PCR products of two genes made with *Pfu*-Ultra DNA polymerase were cloned into the pGEM-T easy vector, and the nucleotide sequence of the PCR-generated insert was then determined with a BigDye terminator cycle sequencing kit for ABI377 PRISM (PerkinElmer Inc., Boston, MA, USA). The inserts were excised from pGEM-T easy using specific restriction endonuclease recognition sites in each primer (EcoRV and XhoI for the PsKP gene and NdeI and XhoI for the PsPGM gene) and ligated into pHXHD treated with EcoRV and XhoI to create pHK-PsKP and pET21a digested with NdeI and XhoI to generate pET-PsPGM.

Purification and characterization of recombinant enzymes. For expression of recombinant PsKP, *E. coli* BL21 CodonPlus(DE3)-RP harboring pHK-PsKP was grown on 500 ml LB medium containing ampicillin (100 μ g/ml) and chloramphenicol (34 μ g/ml) at 37°C for 24 h without the induction step due to the constitutive nature of the expression vector. Similarly, PsPGM was expressed through *E. coli* BL21 Codon-Plus(DE3)-RP harboring pET-PsPGM, which was grown on 500 ml LB medium supplemented with ampicillin (100 μ g/ml) and chloramphenicol (34 μ g/ml) at 37°C. When the optical density of the cell culture reached an absorbance of 0.55 at 600 nm, 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) was added to the cell culture for induction, and the cells were incubated for 24 h at 37°C.

The cells were harvested by centrifugation at 4,000 $\times$ g for 20 min and suspended in lysis buffer (50 mM Na₂HPO₄, 250 mM NaCl, 10 mM imidazole [pH 8.0]). The cell suspensions were disrupted at 4°C by sonication (Sonifier 450; Branson, Danbury, CT, USA; output 4, 6 times for 10 s, constant duty), and cellular debris was removed by centrifugation at 12,000 $\times$ g for 20 min. The crude enzymes were passed through a nickel-nitrilotriacetic acid (Ni-NTA) affinity column (Qiagen Inc., Valencia, CA, USA). The column was washed with washing buffer (50 mM Na₂HPO₄, 300 mM NaCl, and 20 mM imidazole [pH 8.0]), and then the recombinant enzymes were eluted with elution buffer (50 mM Na₂HPO₄, 250 mM NaCl, and 250 mM imidazole [pH 8.0]). The eluted fractions were dialyzed to remove the excess imidazole. Protein concentration was determined by the Bradford reagents kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. The purity and molecular mass of the recombinant proteins were estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a 10% (wt/vol) acrylamide gel. Samples were boiled at 100°C for 5 min in loading buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 14.4 mM β -mercaptoethanol, 25%

glycerol, and 0.1% bromophenol blue) and then centrifuged at 12,000 $\times$ g for 1 min. After electrophoresis, the gel was stained in 0.025% Coomassie blue R-250 and then destained in 10% methanol with 10% acetic acid.

Preparation of β -glucose-1-phosphate. β -Glucose-1-phosphate (β -G1P) was synthesized from trehalose using trehalose phosphorylase from *Anaerocellum thermophilum* (*Caldicellulosiruptor bescii*) by a modification of the method described by Van der Borgh et al. (21). The reaction was performed at 55°C for 18 h with 200 mM trehalose as the substrate dissolved in 200 mM sodium phosphate buffer (pH 7.0). The amount of β -G1P was calculated indirectly by measuring the concentration of glucose generated from the reaction. After the reaction, the remaining trehalose was further hydrolyzed into two glucose molecules by trehalase from *E. coli* DH10B (21). The glucose in the mixture was removed by incubation with baker's yeast (21) at 37°C for 8 h. After elimination of glucose from the reaction, enzymes and proteins secreted from yeast were denatured and removed by boiling for 20 min.

Removal of inorganic phosphate was required in the procedure for β -G1P purification, since it can inhibit the synthetic reaction of kojibiose phosphorylase. Therefore, inorganic phosphate was precipitated by adding equal concentrations of ammonia water and magnesium acetate (22). Finally, a 10-fold concentration of 99% ethanol was added to the mixture containing β -G1P to concentrate the β -G1P, and then centrifugation was performed at 10,000 $\times$ g for 10 min. The pellet was dried at 55°C for 12 h and dissolved in distilled water.

Determination of enzyme activities. The phosphorylase activity of PsKP was assayed using a GLzyme glucose oxidase kit (Shinyang, Seoul, South Korea). The reaction mixture contained 4 mM concentrations of various disaccharides in 80 mM sodium phosphate buffer (pH 6.0) with 0.2 U of enzyme and was incubated at 90°C for 5 min. The reaction mixture was mixed with 900 μ l of GLzyme glucose oxidase solution at 37°C for 15 min to determine the concentration of glucose. The color developed was measured spectrophotometrically at 505 nm. One unit (U) of enzyme activity was defined as the amount of the enzyme that produced 1 μ mol of glucose per min.

The conversion activity of PsPGM was determined by measuring the concentration of G6P transformed from β -G1P using high-performance anion-exchange chromatography (HPAEC). The reaction mixture contained 10 mM β -G1P in 60 mM Britton-Robinson universal buffer (pH 6.0) with 0.44 U of PsPGM and was incubated at 90°C for 5 min. The mixture was stopped by the addition of an equal volume of 150 mM NaOH and filtered onto a 0.2- μ m-pore-size membrane filter (Whatman). The filtered samples were subjected to the HPAEC analysis (see below). One unit of enzyme activity was defined as the amount of the enzyme that produced 1 μ mol of G6P per min.

Effects of temperature and pH. In order to study the influence of pH and temperature on PsKP phosphorolysis activity, enzymatic reactions were carried out using 0.2 U of purified enzyme, and the glucose oxidase method was used to determine the resulting glucose concentration. The relative activity of the enzyme for kojibiose was examined at various pHs between 4.0 and 9.0 using Britton-Robinson universal buffer. The reaction mixture contained 300 mM phosphate to direct the phosphorolysis reaction. The effect of temperature on the enzyme activity was determined at various temperatures ranging from 50 to 95°C in 80 mM sodium phosphate buffer (pH 6.0). Similarly, the effects of pH and temperature on the PsPGM reaction were confirmed by HPAEC analysis.

Assay of substrate specificity of PsKP. The substrate specificity of PsKP for various disaccharides, including trehalose, kojibiose, nigerose, maltose, isomaltose, sucrose, isomaltulose, turanose, cellobiose, and lactose, was investigated with 0.2 U of enzyme in 80 mM sodium phosphate buffer (pH 6.0) at 90°C for 5 min. The concentration of glucose, a phosphorolysis product of the reaction, was determined by using GLzyme glucose oxidase solution as described above.

Thin-layer chromatography. Thin-layer chromatography (TLC) analysis was performed with Whatman (Kent, United Kingdom) K5F silica gel plates. After a TLC plate had been heated at 110°C for 30 min,

FIG 1 (A and B) TLC analysis of reaction products by *Pyrococcus* sp. strain ST04 crude extract with various disaccharides, such as trehalose, kojibiose, nigerose, maltose, and isomaltose (A) and turanose, sucrose and isomaltulose (B). The reaction was performed at 80°C for 10 h. Lane M, G1 to G7 standards; – and +, absence and presence of enzyme, respectively. (C) TLC analysis of kojibiose hydrolysis reaction products for various times. The reaction was carried out with 10 mM kojibiose at 80°C for various intervals. Lane M, G1 to G7 standards; lane C, 0 h; lane 1, 4 h; lane 2, 8 h; and lane 3, 12 h.

0.5- μ l aliquots of the reaction mixture were spotted onto a K5F silica gel plate and developed with a solvent system of isopropanol-ethyl acetate-water (3:1:1, vol/vol/vol). The developed TLC plate was dried in a hood and then visualized by soaking quickly in 0.3% (wt/vol) *N*-(1-naphthyl)-ethylenediamine and 5% (vol/vol) H_2SO_4 in methanol. The plate was dried and heated in an oven for 10 min to observe the reaction spots.

High-performance anion-exchange chromatography (HPAEC). The reaction mixtures were added to an equal volume of 150 mM NaOH and filtered using a 0.2- μ m-pore-size membrane filter. For HPAEC, a CarboPac PA-1 column (0.4 by 25 cm; Dionex, Sunnyvale, CA, USA) and an electrochemical detector (ED40; Dionex) were used. Two buffers, A (150 mM NaOH) and B (150 mM NaOH and 500 mM sodium acetate), were used for the elution of the sample, with 100 to 0% gradient of buffer B for 60 min at a flow rate of 1.0 ml/min.

Kinetic analysis. A kinetic analysis of the phosphorylation reaction was performed using the GLzyme glucose oxidase kit. Reaction mixtures containing various concentrations of kojibiose (between 1 and 10 mM) and phosphate (between 1 and 10 mM) were incubated at 90°C after adding 0.2 U of PsKP. Twenty microliters of final reaction mixtures were mixed with 190 μ l of GLzyme glucose oxidase. After incubation at 37°C for 10 min, the color developments were measured spectrophotometrically at 505 nm with an Infinite 200 PRO reader (TECAN, Männedorf, Switzerland). The kinetic parameters of the reaction were calculated with the following equation for an ordered bi-bi mechanism:

$$v = \frac{V_{\max}[A_0][B_0]}{(K_{iA}K_{mB}) + (K_{mB}[A_0]) + (K_{mA}[B_0]) + ([A_0][B_0])}$$

where K_{mA} and K_{mB} are the theoretical K_m values of substrates A (kojibiose) and B (phosphate), respectively, where the concentrations of the other substrates are infinity. K_{iA} is the K_i value of substrate A, where [B] is 0. To determine the kinetic parameters of the synthetic reaction, the initial rates of production of kojibiose were measured by HPAEC analysis. The reaction mixtures containing various concentration of glucose (between 1 mM and 10 mM) with 26 mM β -G1P were incubated at 90°C for 2 min. The kinetic parameters of the reaction were calculated by a Lineweaver-Burk plot.

The kinetic parameters of the conversion reaction for PsPGM were investigated with various concentrations of β -G1P (between 0.78 mM and 6 mM). The reactions were carried out with 0.44 U of PsPGM at 90°C for 5 min. The initial rates of production of G6P were measured by HPAEC analysis. The kinetic parameters of the reaction were calculated using the

Michaelis-Menten equation of GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

Amino acid sequence analysis of PsKP. Nucleotide sequences of various DPases belong to the GH65 family were obtained from the National Center for Biotechnology Information (NCBI) and Carbohydrate-Active Enzymes database (CAZy). Sequence homologies of deduced amino acids were analyzed using BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple alignments were carried out using ClustalW2 (23). The phylogenetic tree was constructed by the neighbor-joining method of the MEGA 4.0 program (24) with the following sequences: bacterial kojibiose phosphorylases from *Caldicellulosiruptor saccharolyticus* (ABP66077) and *Thermoanaerobacter brockii* (BAB97300); bacterial maltose phosphorylases from *Lb. sanfranciscensis* (CAA11905), *Lactobacillus acidophilus* NCFM (AAV43670), *Bacillus* sp. strain RK-1 (BAC54904), and *Paenibacillus* sp. strain SH-55 (BAD97810); bacterial trehalose phosphorylases from *Geobacillus stearothermophilus* (BAC20640), *C. saccharolyticus* (ABP66082), *Carboxydibrachium pacificum* (ZP_05091985) and *Th. brockii* (BAB97299); eukaryotic trehalases from *Metarhizium acridum* (ABB51158), *Aspergillus nidulans* (EAA66407), and *Saccharomyces cerevisiae* (CAA58961); nigerose phosphorylase from *Cl. phytofermentans* (ABX42243); 3-O- α -glucopyranosyl-L-rhamnose phosphorylase from *Cl. phytofermentans* (ABX41399); and trehalose-6-phosphate phosphorylase from *Lactococcus lactis* subsp. *lactis* (AAK04526).

RESULTS

Disaccharide hydrolysis pattern of *Pyrococcus* sp. strain ST04.

To investigate disaccharide hydrolysis by *Pyrococcus* sp. strain ST04, the crude extracts of *Pyrococcus* sp. strain ST04 cells containing 0.2 mg/ml of total proteins were incubated with various disaccharides at 80°C. Maltose, isomaltose, kojibiose, and nigerose were cleaved into two molecules of glucose, and isomaltulose was slightly hydrolyzed to glucose and fructose, whereas trehalose, sucrose, and turanose were not degraded (Fig. 1A and B). The hydrolysis of maltose and isomaltose was the result of α -glucosidase activity, since it is known to cleave maltose, isomaltose, and panose into two glucose units (25). However, this is the first report of kojibiose and nigerose hydrolysis by a *Pyrococcus* species. The hydrolysis of kojibiose produced an unknown compound other than glucose that increased in abundance with incubation time (Fig. 1C). The kojibiose hydrolysate was subjected to HPAEC

FIG 2 Comparison analysis of ORFs of the disaccharide phosphorylase-related cluster of *Pyrococcus* sp. strain ST04 with those from other archaeal strains, including *Pyrococcus horikoshii* OT3 and *Thermococcus barophilus* MP. Amino acid identities (percentages) are indicated between homologous genes. Locus tag numbers of homologs in the different strains are given. The flanking numbers are the starting and ending positions of target gene clusters.

analysis to identify this unknown compound. It was shown to be β -G1P (data not shown), suggesting that DPase was responsible for the degradation of kojibiose.

Identification of a catabolic gene cluster containing disaccharide phosphorylase. Analysis of the whole genome of *Pyrococcus* sp. strain ST04 (20) revealed the presence of a unique catabolic gene cluster containing a putative DPase. This cluster is located between positions 1488209 and 1494318 of the genome and consisted of seven open reading frames (ORFs) (Py04_1502 to Py04_1508). Interestingly, homologous gene clusters were also found in the genomes of *Thermococcus barophilus* MP and *Pyrococcus horikoshii* OT3 in the *Thermococcales*, and the organizations of their clusters exactly matched those of *Pyrococcus* sp. strain ST04 (Fig. 2). The clusters can be divided into three parts based on function. The first part is composed of sugar transporter-related proteins, including two transmembrane proteins, MalF-like and MalG-like maltose/maltodextrin ABC transporter (Py04_1506 and Py04_1507), sugar-binding protein (Py04_1505), and ABC type-permease protein (Py04_1508). The second part is a transcriptional regulator (Py04_1504), which may control this sugar-metabolic pathway gene cluster. The third part consists of two ORFs, Py04_1502 and Py04_1503, that are predicted to encode a DPase and a hypothetical protein containing an HAD-like domain.

The gene Py04_1502, for DPase, is 2,115 bp long and encodes a protein of 704 amino acids with a calculated molecular mass of 82,127.83 Da. SignalP 4.0 did not detect the presence of a membrane secretory signal peptide sequence, suggesting that this protein is an intracellular enzyme (26). Although it was confirmed as a kojibiose phosphorylase (designated PsKP, for *Pyrococcus* sp. ST04 kojibiose phosphorylase) in this study, it showed low sequence homology with other established DPases, such as kojibiose phosphorylases from *Th. Brockii* (37%) (27) and *C. saccharolyticus* (37%) (28) and trehalose phosphorylases from *Th. Brockii* (30%) (29), *Ca. pacificum* (27%) (30), and *C. saccharolyticus* (28%) (28). In members of the *Thermococcales*, there are some PsKP homologs, such as maltose phosphorylase from *T. barophilus* (53%)

and hypothetical protein PH0746 from *P. horikoshii* OT3 (55%) (Fig. 2). However, their catalytic properties are uncharacterized.

The protein encoded by Py04_1503 is composed of 234 amino acid residues with an estimated molecular mass of 26,396 Da. BLASTP analysis revealed that this gene product has high sequence homology with hypothetical proteins of *P. horikoshii* OT3 (ADT83253) (60%) and *T. barophilus* MP (BAA29840) (61%), and contained a haloacid dehalogenase domain (HAD). However, it displayed no significant identity with any reported enzymes. Even though the function of Py04_1503 could not be predicted from the sequence, we predicted that it participated in disaccharide metabolism given its proximity to the putative PsKP gene. The majority of HAD family enzymes are phosphatases, namely, ATPases and phosphomutases (31); therefore, we focused primarily on mutase activity, and we named the product of Py04_1503 *Pyrococcus* sp. strain ST04 β -phosphoglucomutase (PsPGM).

Cloning and expression of the PsKP and PsPGM genes in *E. coli*. To identify the enzymatic properties of PsKP and PsPGM, each gene was isolated and amplified from genomic DNA of *Pyrococcus* sp. strain ST04 using PCR. Both constitutive and inducible vectors (pHCXHD and pET-21a, respectively) were used to express PsKP and PsPGM genes. The PsKP gene was more effectively expressed in pHCXHD, while the PsPGM gene was more effectively expressed in pET-21a. Both recombinant proteins were thermostable during heat treatment at 70°C for 20 min, while the heat-labile proteins in *E. coli* lysates were all denatured (Fig. 3). Ni-NTA affinity chromatography was used to purify the recombinant proteins, which each contained a 6 $\times$ His tag on their C termini. Recombinant PsKP was purified 6.3-fold with a yield of about 9.2% from the cell extract. It was a major band on an SDS-PAGE gel with an estimated molecular mass of 80 kDa (Fig. 3A), which closely matched the predicted mass of the protein with the added 6 $\times$ His tag. Similarly, the recombinant PsPGM was a single band on an SDS-PAGE gel with an estimated molecular mass of 25 kDa, which closely matched its predicted mass (Fig. 3B).

Kojibiose and nigerose phosphorolysis activity of PsKP. The enzymes in GH65 are known to have a phosphorolysis activity for

FIG 3 SDS-PAGE analysis of purified recombinant PsKP (A) and PsPGM (B) expressed in *E. coli* BL21 CodonPlus(DE3)-RP. Lane M, protein size marker; lane 1, crude cell extract; lane 2, crude cell extract after heat treatment at 80°C for 20 min; lane 3, purified recombinant enzyme after Ni-NTA chromatography.

disaccharides such as trehalose, kojibiose, nigerose, and maltose. The substrate specificity of PsKP was determined with 10 mM concentrations of various disaccharides at 80°C for 12 h (Fig. 4). The substrates used were in two groups. One group contained α -1,1 (trehalose), α -1,2 (kojibiose), α -1,3 (nigerose), α -1,4 (maltose), and α -1,6 (isomaltose) glycosidic linkages between two glucose units. The other group included α -1,2 (sucrose), α -1,3 (turanose), and α -1,6 (isomaltulose) linkages between the glucose and fructose units. The result of TLC analysis showed that PsKP had strong phosphorolysis activity on kojibiose, weak activity on nigerose, and no activity on other disaccharides (Fig. 4A). The phosphorolysis activity of PsKP was determined by the measurement of the amount of glucose released from kojibiose. The specific activity for kojibiose was 10.7 ± 0.21 U/mg. Nigerose was also cleaved into glucose and β -G1P, but the specific activity of nigerose was much lower than that of kojibiose (Table 1). In the presence of inorganic phosphate, we verified the production of β -G1P from kojibiose using HPAEC analysis. These results indicate that PsKP had kojibiose phosphorolysis activity, and it was designated kojibiose phosphorylase.

The effect of pH on this activity showed that over 90% of it

TABLE 1 Specific activity of PsKP on various disaccharides (10 mM) at 90°C

Substrate	Linkage	Sp act (U/mg) ^a	Relative activity (%)
Trehalose	α -1,1	ND	
Kojibiose	α -1,2	10.7 ± 0.21	100
Nigerose	α -1,3	$(2.60 \pm 0.2) \times 10^{-2}$	0.23
Maltose	α -1,4	$(6.31 \pm 0.2) \times 10^{-4}$	<0.01
Isomaltose	α -1,6	$(2.89 \pm 0.3) \times 10^{-4}$	<0.01
Cellobiose	β -1,4	ND	

^a ND, not detected.

remained between pH 4.0 and 6.0, with optimum activity at pH 5.0. The activation energy was calculated as 14.72 ± 0.73 kJ/mol. PsKP was highly thermostable at 100°C, with over 40% of the activity remaining after 2 h at 100°C (data not shown). It showed higher thermal stability than other DPases, with a half-life of 71 h and 1.9 h at 95°C and 100°C, respectively. The metal ion effect on the phosphorolysis activity of PsKP was carried out with various metal ions and reagents. In the presence of 1 mM $MnCl_2$, activity of PsKP decreased to 66% of maximum activity. The metal ions, $ZnCl_2$, $CdCl_2$, and $CuCl_2$ elevated the phosphorolysis activity of PsKP 36%, 33%, and 40%, respectively. PsKP did not require metal ions for the phosphorolysis reaction, and 1 mM EDTA did not affect its activity (data not shown).

To determine the kinetic parameters of PsKP, glucose-releasing activity from kojibiose was measured at 90°C. Generally, the phosphorolysis reaction needs two substrates, kojibiose and inorganic phosphate. The kinetic parameters were determined in the range of the kojibiose and phosphate concentrations within 10 mM. This indicated that the phosphorolysis reaction of PsKP follows a sequential bi-bi mechanism, as reported for other inverting phosphorylases (32). The K_m values of kojibiose (K_{mA}) and phosphate (K_{mB}) were 2.53 ± 0.21 mM and 1.34 ± 0.04 mM, respectively. The k_{cat} value was calculated to be $2,264 \pm 24$ min⁻¹, and the catalytic efficiencies (k_{cat}/K_m) of kojibiose and phosphate were determined as 897 ± 86 and $1,683 \pm 73$ mM⁻¹ min⁻¹, respectively (Table 2). Generally, phosphorylase has the reverse activity

FIG 4 TLC analysis of the phosphorolysis reaction with trehalose, kojibiose, nigerose, maltose, and isomaltose (A) and turanose, sucrose, isomaltulose, cellobiose, and lactose (B) as substrates. Reactions were performed with 0.2 U of PsKP and a 10 mM concentration of each substrate at 80°C for 12 h. Lane M, G1 to G7 standards; – and +, absence and presence of enzyme, respectively.

TABLE 2 Kinetic parameters of PsKP for kojibiose and phosphate as substrates

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \text{mM}^{-1}$)
Kojibiose	2.53 ± 0.21	$2,264 \pm 24$	897 ± 86
Phosphate	1.34 ± 0.04	$2,264 \pm 24$	$1,683 \pm 73$

of phosphorolysis, known as synthetic activity. PsKP exhibited synthesis of kojibiose and kojiligosaccharides, which are glucose polymers linked by α -1,2-glycosidic bonds (33). In the synthesis reaction, PsKP had low affinity for glucose. The K_m value of glucose is 36.8 ± 4.61 mM in the presence of a concentration of β -G1P (26 mM) high enough to act as a glucosyl donor in the synthesis reaction. It was 15-fold higher than that of kojibiose in phosphorolysis reactions.

Conversion of β -G1P to G6P by PsPGM. While PsKP catalyzes the phosphorolysis of kojibiose and produces glucose and β -G1P, the resulting β -G1P must be converted into G6P for glycolysis. This reaction is catalyzed by β -phosphoglucumutase. We predicted that Py04_1503 (encoding PsPGM) encodes the protein responsible for the transformation of β -G1P to G6P, since some HAD family enzymes are phosphomutases (31). To confirm the mutase function of PsPGM, the converting activity of PsPGM was verified by quantifying the G6P produced from β -G1P using HPAEC analysis (Fig. 5). The results show that PsPGM transforms β -G1P to G6P. β -Phosphoglucumutases typically use MgCl_2 and glucose-1,6-bisphosphate (G1,6bP) as cofactors (34). The conversion activity of *Pyrococcus* sp. strain ST04 β -phosphoglucumutase was absent without MgCl_2 but was present without G1,6bP.

In general, phosphoglucumutases, including both α -phosphoglucumutase and β -phosphoglucumutase, need metal ions as cofactors and have a highly conserved metal-binding site on their N termini. From the alignment with other phosphoglucumutase, a conserved metal binding site was found in PsPGM despite overall low sequence identity (<40%). The effect of various concentrations of MgCl_2 on PsPGM was investigated, indicated that 6 mM MgCl_2 is the optimal concentration for phosphoglucumutase activity (data not shown).

The specific activity of the conversion reaction was performed with 10 mM substrate, 6 mM MgCl_2 , and 0.36 μg of PsPGM. The conversion activity from β -G1P to G6P was 46.8 ± 3.66 U/mg, and the reverse conversion activity from G6P to β -G1P was 3.51 ± 0.13 U/mg. This indicates that the major reaction of PsPGM directs the production of G6P from β -G1P. The kinetic parameters were determined in the range of the β -G1P concentrations between 0.75 and 6 mM using the Michaelis-Menten equation. The K_m value of PsPGM for β -G1P was 2.08 ± 0.63 mM, and the catalytic efficiency (k_{cat}/K_m) of PsPGM was $5.44 \times 10^2 \pm 0.52 \times 10^2 \text{ min}^{-1} \text{mM}^{-1}$. In the presence of 6 mM MgCl_2 , PsPGM converted β -G1P to G6P. Conversion activity was observed between pH 4.0 and pH 9.0 with an optimum at pH 6.0, and its activation energy is 107.3 ± 5.13 kJ/mol (data not shown). PsPGM was highly thermostable, with optimum conversion activity at 95°C, which is the highest temperature of activity reported for β -phosphoglucumutase.

DISCUSSION

It was reported previously that *Pyrococcus* and *Thermococcus* species utilize various carbohydrate substrates, such as starch, by us-

ing a series of gene products containing amylopullulanase and a maltodextrin transporter (4). Maltodextrins imported into the cell were further degraded to glucose by 4- α -glucanotransferase and α -glucosidase or transformed to α -glucose-1-phosphate via α -glucan phosphorylase (3, 35). In *Thermococcus* sp. strain B1001 and *Archaeoglobus fulgidus*, genes for two cyclodextrin-utilizing enzymes, cyclodextrin glucanotransferase (CGTase) and cyclodextrinase (CDase), together with an ABC transporter cluster were found in the genome. In these strains, cyclodextrin is formed by CGTase using starch, then assimilated into the cell through an ABC transporter system, and completely hydrolyzed into glucose and maltose by CDase (2, 36). Until now, the utilization of other disaccharides, such as kojibiose, in the archaea has not been reported. Kojibiose was isolated from honey and dextran derived from *Betacoccus arabinosaceus* (37, 38), but Sugisawa and Edo also reported that it was made by heating glucose at 150°C (39). This suggests that kojibiose might be a possible carbon source for hyperthermophilic archaea in hot environments.

The pathway resembles the maltose metabolic pathway that depends on a maltose phosphorylase and a β -phosphoglucumutase in *L. lactis* and *Lb. acidophilus* (14). A similar gene cluster containing nigerose phosphorylase was found in *Cl. phytofermentans*. It was suggested that the source of nigerose was nigeran, an unbranched glucan found in the hyphal wall of fungi (1). In *Pyrococcus* sp. strain ST04, the kojibiose was absorbed by the ABC transporter (Py04_1505 to Py04_1508) and utilized by two enzymes, kojibiose phosphorylase and β -phosphoglucumutase, as shown in Fig. 6. The kojibiose catabolic gene cluster was probably regulated by a TrmB family transcriptional regulatory protein (Py04_1504). Members of the TrmB family can be sugar-specific transcriptional regulators of the trehalose/maltose ABC transporter (15). This kojibiose catabolic pathway described here for *Pyrococcus* sp. strain ST04 is the first identified in archaea.

Although PsKP has low homology with the previously characterized kojibiose phosphorylases from *C. saccharolyticus* (28) and *Th. Brockii* (27), phylogenetic analysis of DPases in GH65 revealed that PsKP is located close to the bacterial kojibiose phosphorylase subgroup (Fig. 7). Multiple sequence alignments with other DPases show that Asp316 and Glu455 of PsKP correspond with the catalytic residues of maltose phosphorylase from *Lactobacillus brevis* (Asp359 and Glu487) (27, 40). The amino acid residues closely related to catalytic residues were ³¹⁴FWDTEIY³²⁰ and

FIG 5 HPAEC analysis of conversion of β -G1P to G6P by PsPGM.

FIG 6 Proposed model of kojibiose catabolic pathway in *Pyrococcus* sp. strain ST04. One-step and multistep reactions are indicated that by solid arrows and dotted arrows, respectively.

⁴⁵²GADEYHEH⁴⁵⁹ (underlining indicates catalytic residues of PsKP), which were similar to those of other kojibiose phosphorylases from *C. saccharolyticus* and *Th. Brockii* but different from maltose phosphorylase and trehalose phosphorylase (data not shown). These results suggest that archaeal PsKP has a distinct substrate binding site for kojibiose. This was also confirmed by the narrow substrate specificity of PsKP. Only kojibiose and nigerose were phosphorylated by the action of PsKP. The activity of kojibiose phosphorylase was approximately 400-fold higher than that of nigerose phosphorylase (relative activity, 0.23%). Similar substrate specificity was also observed in kojibiose phosphorylases from *C. saccharolyticus*. The enzyme with activity for nigerose showed only 0.73% activity for kojibiose (28). Another kojibiose phosphorylase from *Th. Brockii* hydrolyzed only kojibiose (41). The level of nigerose phosphorylase was determined by the shape of substrate binding site of the each enzyme, since α -1,2-linkage and α -1,3-linkage share similar structural requirements (1).

The PsKP has the highest optimum temperature and thermostability among DPases belonging to GH65 (data not shown). The optimal temperature of PsKP was 90°C, while those of kojibiose phosphorylase from *C. saccharolyticus* and trehalose phosphorylase from *Ca. pacificum* DSM 12653 were reported as 85°C and 80°C, respectively (28, 30). The half-life of PsKP at 95°C was 72 h, which corresponds to the high optimal growth temperature of *Pyrococcus* sp. strain ST04.

Physiologically, the main direction of the PsKP reaction is phosphorylase producing β -G1P and glucose from kojibiose and its derivatives but not synthesizing kojioligosaccharides. The K_m values for kojibiose and phosphate were 2.53 ± 0.21 and 1.34 ± 0.04 mM, respectively, which was much lower than that of glucose (20-fold). This is similar to the pattern observed with kojibiose phosphorylase from *Th. Brockii* (41). Inorganic phosphate is one of the essential compounds for this reaction (11, 42).

Unlike α -G1P generated from glucokinase or α -glucan phosphorylase, β -G1P was not converted to G6P by α -phosphoglucosylmutase. To convert β -G1P to G6P, an enzyme having β -phosphoglucosylmutase activity was needed. Previously, the properties of β -phosphoglucosylmutases from *Thermotoga maritima* (43), *L. lactis* (34), *Lb. brevis* (44), *Lb. delbrueckii* subsp. *lactis* (45), *Neisseria meningitidis* (46), *Neisseria perflava* (47), *B. subtilis* (48), and *Euglena gracilis* (49) have been characterized. Although these proteins have low homology with PsPGM (data not shown), they commonly have a conserved core domain catalytic scaffold of the phosphatase branch of the HAD family (43, 50). Multiple alignments of PsPGM together with various β -phosphoglucosylmutases also indicate that their core amino acid residues on the metal ion binding site and substrate binding site were highly conserved (data not shown).

The K_m value of PsPGM is higher than that of other β -phosphoglucosylmutases. The K_m values of β -phosphoglucosylmutase from

FIG 7 Phylogenetic tree of characterized enzymes belonging to the GH65 family. Multiple alignments were carried out using ClustalW2. Shown are bacterial kojibiose phosphorylases from *Caldicellulosiruptor saccharolyticus* (ABP66077) and *Thermoanaerobacter brockii* (BAB97300); bacterial maltose phosphorylases from *Lactobacillus sanfranciscensis* (CAA11905), *Lactobacillus acidophilus* NCFM (AAV43670), *Bacillus* sp. strain RK-1 (BAC54904), and *Paenibacillus* sp. SH-55 (BAD97810); bacterial trehalose phosphorylases from *Geobacillus stearothermophilus* (BAC20640), *Caldicellulosiruptor saccharolyticus* (ABP66082), *Carboxydibrachium pacificum* (ZP_05091985), and *Thermoanaerobacter brockii* (BAB97299); eukaryotic trehalases from *Metarhizium acridum* (ABB51158), *Aspergillus nidulans* (EAA66407), and *Saccharomyces cerevisiae* (CAA58961); nigerose phosphorylase from *Clostridium phytofermentans* (ABX42243); 3-O- α -glucopyranosyl-L-rhamnose phosphorylase from *Clostridium phytofermentans* (ABX41399); and trehalose-6-phosphate phosphorylase from *Lactococcus lactis* subsp. *lactis* (AAK04526).

B. subtilis and *L. lactis* were 0.004 mM and 0.0146 mM, respectively (48, 50). This distinct property may result from the absence of G1,6biP as a cofactor. Usually, G1,6biP is used to activate a catalytic residue of β -phosphoglucomutase. In the absence of G1,6biP, PsPGM could convert β -G1P to G6P, suggesting that PsPGM also has G1P phosphodismutase activity which catalyzes the G1P to glucose through the forming G1,6biP (48). The decrease of activity in the absence of G1,6biP has also been observed with *Acetobacter xylinus* α -phosphoglucomutase. The activity without G1,6biP was measured at 70% of the activity with G1,6biP (51).

In summary, *Pyrococcus* sp. strain ST04 has a kojibiose catabolic gene cluster that is rarely found in archaea. It possesses two carbohydrate-active enzymes, kojibiose phosphorylase (Py04_1502) and β -phosphoglucomutase (Py04_1503). In the presence of kojibiose, PsKP (Py04_1502) catalyzes the phosphorolysis reaction, producing glucose and β -G1P. Then PsPGM (Py04_1503) converts β -G1P to G6P for use in glycolysis. It may be used to generate energy without consumption of ATP. Generally, glucose is phosphorylated to G6P by glucokinase with consumption of ADP or ATP.

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