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Cloning and Characterization of Phosphoglucomutase and Phosphomannomutase Derived from *Sphingomonas chungbukensis* DJ77

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The enzymes phosphoglucomutase (PGM) and phosphomannomutase (PMM) play an important role in the synthesis of extracellular polysaccharide. By colony hybridization of the fosmid library of *Sphingomonas chungbukensis* DJ77, an open reading frame (ORF-1) of 1,626 nucleotides, whose predicted product is highly homologous with other PGM proteins from several bacterial species, was identified. An additional open reading frame (ORF-2) of 1,437 nucleotides was identified, and its encoded protein shows a high level of similarity with the PGM/PMM protein family. The two genes were cloned into a bacterial expression vector pET-15b (+) and expressed in *Escherichia coli* as fusion proteins with $(His)_6$-tag. Both recombinant proteins (designated as SP-1 and SP-2 for ORF-1 and ORF-2, respectively) exhibited PGM and PMM activities. The molecular masses of subunits of SP-1 and SP-2 were estimated to be around 58 and 51 kDa from SDS–PAGE, respectively. However, molecular masses of SP-1 and SP-2 in their native condition were determined to be approximately 59.5 and 105.4 kDa, according to non-denaturing PAGE, respectively. The SP-1 protein has a preference for glucose-1-phosphate rather than mannose-1-phosphate, while the preferred substrate of SP-2 is mannose-1-phosphate. Thus, the existence of two proteins with bifunctional PGM/PMM activities was first found *S. chungbukensis* DJ77.

Key words: extracellular polysaccharide, gene cloning, phosphoglucomutase, phosphomannomutase, *Sphingomonas chungbukensis DJ77*, synthesis.

Sphingomonas chungbukensis DJ77 was isolated from contaminated freshwater sediment in Daejeon, Korea (*1*). Like many species in the genus *Sphingomonas* (*2*), this strain produces a large quantity of extracellular polysaccharide (EPS) (*1*), creating characteristic high viscosity in culture media. To exploit EPS synthesis by DJ77 for the purpose of metabolic engineering, it is necessary to fully characterize the enzymes involved in EPS biosynthesis.

Among the enzymes predicted to be involved in the EPS synthetic pathway, phosphoglucomutase (PGM) is speculated to play an important role and is an ideal target for metabolic engineering. PGM catalyses the reversible interconversion between glucose-6-phosphate (G6P) and glucose-1-phosphate (G1P) by functioning at the branch point in glucose metabolism in which G6P enters glycolysis, while G1P enters the metabolic pathway leading to the sugar precursors of polysaccharide synthesis (e.g. UDP–glucose, UDP–glucoronic acid, UDP–galactose and dTDP–rhamnose) (*3*).

Another enzyme, phosphomannomutase (PMM), is also predicted to participate in EPS synthesis in *S. chungbukensis* DJ77. PMM reversibly converts mannose-6-phosphate (M6P) into mannose-1-phosphate (M1P) that reacts with GTP to form the sugar precursor GDP–D-mannose by the action of GDP–mannose pyrophosphorylase. It has been reported that in some EPS producing species, the PMM and PGM activities are coupled with the same protein, i.e. a bifunctional PGM/PMM (*3*).

In this study, the genes encoding proteins having both PGM and PMM activities from *S. chungbukensis* DJ77 were cloned, overexpressed in *E. coli*, and the enzymes were fully characterized.

MATERIALS AND METHODS

Bacterial Strains and General Reagents—The bacterial strains and plasmids used in this study are listed in Table 1. *Sphingomonas chungbukensis* and the fosmid library were prepared in the laboratory of Dr Young-Chang Kim (*1*). *Sphingomonas chungbukensis* was prepared in the laboratory of Dr Young-Chang Kim as described previously (*1*). Fosmid library construction was carried out according to the methods reported previously (*4*, *5*) using CopyControl™ Fosmid Library Production Kit (Epicentre, Madison, USA) The restriction enzymes and T4 ligase were purchased from Roche

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Table 1. **Bacterial strains and plasmids used in this study.**

Strain or plasmid	Relevant properties	Sources or references
Strains		
S. chungbukensis DJ77	EPS producing strain	Dr Y.C. Kim (*1*)
E. coli XL1Blue	*E. coli* strain for cloning	Stratagene
E. coli BL21(DE3)	*E. coli* strain for overexpression	Novagen
Plasmids		
pGEM-T	Sequencing vector	Promega
pBluescript II SK(+)	Cloning vector	Stratagene
pET-15b	Expression vector	Novagen
FA494	Fosmid containing pgm sequence	Dr Y.C. Kim (*1*)
FB186	Fosmid containing pmm sequence	Dr Y.C. Kim (*1*)

(Mannheim, Germany), while Taq polymerase and other components for PCR were purchased from Bioneer Inc. (Daejeon, Korea). PCR primers were synthesized by Genotech (Daejeon, Korea). Glucose-1-phosphate, glucose-1, 6-diphosphate, mannose-1-phosphate, phosphomannose isomerase, phosphoglucose isomerase and phosphoglucose dehydrogenase were obtained from the Sigma Chemical Co. (St Louis, USA). All other reagents were purchased from Sigma or other commercial companies.

DNA Manipulations—Plasmid DNA was purified using the Qiaprep spin kit of Qiagen (Valencia, USA). PCR amplification was performed using a thermocycler, and DNA sequencing carried out at Marcrogen (Seoul, Korea). DNA restriction digestion, cloning and transformation were carried out by established protocols (*6*).

Probe Preparation and Colony Hybridization—To clone the genes encoding PGM and PMM found in *S. chungbukensis* DJ77, two primers were designed using the conserved regions of the phosphohexomutase family determined in the database (*7*, *8*). The two primers pgmDJ-1(5′-GATGGAGACACTGTTCGACT-3′) and pgmDJ-2(5′-AAAATAATTTGCCTGAACGA-3) are the sequences of the active site region and the substrate specificity region, respectively. Each PCR reaction mixture of 50 μl contained 5 μl of 10× Taq DNA polymerase buffer with $MgCl_2$, 2.5 mM dNTPs, 10 pmol of each primer, 1 U of Taq polymerase and the genomic DNA template. PCR amplification was performed under the following conditions: 30 cycles consisting of 1 min at 94°C, 1 min at 49°C and 1 min at 72°C. The PCR product was cloned into the pGEM-T vector for sequencing.

The probe was end-labelled with DIG-11-dUTP using the DIG-DNA labelling and detection kit purchased from Roche Applied Science (Indianapolis, USA). The DIG-labelled probe was used to screen the *S. chungbukensis* DJ77 fosmid library of 1,500 colonies. Colony hybridization was carried out according to a typical protocol suggested by the manufacturer, and the positive signal detected by chemiluminescence using CDP Star (Roche Applied Science) as a substrate.

Construction of Expression Vectors—The primer pgm1 (5′-AAAGGATCCCATGATACAGACCGTCTCG-3′) and the primer pgm2 (5′-AAAAGGATCCTCAGGTGATGACGCTG GG-3′) were used to amplify the ORF-1. The primer pmm1 (5′-AAAAGGATCCCATGGCCCACCGCTTCCAT-3′) and the primer pmm2 (5′-AAAAGGATCCTCACGCGTTGGGA GACTCT-3′) were used for amplification of ORF-2. The underlined sequences indicate the BamHI restriction site. Genomic DNA was used as a template for PCR. The BamHI-digested PCR products were inserted into the pET-15b expression vector to produce the new constructs pSP-1 and pSP-2 corresponding to ORF-1 and ORF-2, respectively. The nucleotide sequences of SP-1 (pgm) and SP-2 (pmm) reported in this article were submitted to the GeneBank with accession numbers EU128530 and EU128531, respectively.

Expression and Purification of Recombinant Proteins—To express the proteins, pSP-1 and pSP-2 constructs were transformed into the *Escherichia coli* BL21 (DE3). The transformed *E. coli* strains were grown in LB medium (Difco Laboratories, Detroit, USA) containing 50 mg/l ampicillin at 37°C until the absorbance of the culture medium at 600 nm reached 0.6. IPTG was then added to a final concentration of 0.1 mM for the induction, followed by an additional period of cultivation for 5 h at 30°C. Cells were harvested by centrifugation at 5,000*g* for 20 min. The cell pellet was resuspended in binding buffer containing 50 mM Tris (pH 8.0), 0.5 M NaCl. The cell suspension was lysed by sonication, on ice, and the soluble fraction was obtained by centrifugation of the cell lysates at 20,000*g* for 30 min. The supernatant was applied to a column that was pre-loaded with a nickel-chelating resin. The protein purification procedure was conducted as described by the manufacturer's protocol for Qiagen. Purified protein was analysed on SDS–PAGE and non-denaturing PAGE. Protein concentration was estimated by the Bradford method (*9*) using bovine serum albumin as standard.

Enzyme Assays—The activities of PGM and PMM were assayed under the conditions described previously (*2*, *10*). All assays were based on $NADP^+$ reduction in a coupled reaction at 37°C. The activity was monitored via the increase of absorbance at 340 nm on a Beckman DU-650 spectrophotometer. The specific activity is the number of enzyme units per microgram of protein. One unit of enzyme activity is defined as the amount of enzyme required to reduce 1 μmol $NADP^+$ per minute in these assay conditions. PGM activity was measured in the conversion from G1P to G6P with phosphoglucose dehydrogenase (ZWF) as the coupling enzyme. In the PMM assay, M1P was converted into M6P, and then the reaction was coupled with three enzymes: ZWF, phosphoglucose isomerase and phosphomannose isomerase. The standard reaction mixture contained 50 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 10 μM glucose 1, 6-diphosphate, 1 mM $NADP^+$, 1 U of each coupled enzyme and purified protein. Kinetic constants for the PMM and PGM activities were calculated by non-linear fitting of the data into the Michaelis–Menten equation (Eq. 1) using the Origin 6.0 program. The effect of metal ions on PGM activity was determined by adding 2 mM of $MgCl_2$, $CoCl_2$, $CaCl_2$ and $NiCl_2$ into the assay mixture.

The effect of pH on PGM and PMM activities was investigated by performing the assays in 50 mM buffer of 2-(N-Morpholino)ethanesulfonic acid (MES) (5.5–6.5), 3-(N-Morpholino)propanesulfonic acid (MOPS) (6.5–8.0) and {[Tris(hydroxymethyl)methyl]amino}propanesulfonic acid (TAPS) (8.0–9.5).

$$v = \frac{V_{max}}{(1+K_m/[S])} \quad (1)$$

RESULTS

Cloning ORF-1 and ORF-2 Genes—The probe sequence of 800 nucleotides amplified by primer pgmDJ-1 and pgmDJ-2 contained an incomplete ORF and showed high homologies with the PGM and PMM sequences in the database. Colony hybridization using this probe against the genomic fosmid library of DJ77 revealed five positive clones: FA311, FA381, FA463, FA494 and FA574. The FA494 clone was selected by the analysis of PCR products. Serial subcloning of FA494 into pBluescript II SK(+) vector led to the identification of pFA#34 subclone containing a 3,000 bp BamHI fragment that harboured an open reading frame, designated as ORF-1. This ORF consists of 1,626 nucleotides and starts at the rare GTG start codon. The deduced amino acid sequence showed strong homology with other PGMs: 69% identity and 81% similarity with PGM from *Mesorhizobium loti* (NCBI accession no.: AAK58597) (*11*), 67% identity and 81% similarity with PGM from *Agrobacterium tumefaciens* (NCBI accession no.:NP_356570) (*12*), 50% identity and 68% similarity with rabbit PGM (NCBI accession no.: IC4G_B) (*13*). The three highly conserved domains of phosphohexomutase (*3*) (Fig. 1) were found in ORF-1, and the sequence in the sugar binding domain (CGEES) was specific to the PGM subclass. Thus, these data suggest that ORF-1 likely encodes the protein that has the PGM activity.

Screening of the incomplete genomic database of DJ77 revealed that a sequence of fosmid FB186 is homologous to the N-terminal sequence of PMM and the PGM/PMM protein identified in the database. Sequencing extension downstream from the 3′ end of this ending sequence for FB186 revealed a complete open reading frame of 1,437 nucleotides, designated as ORF-2. The amino acid sequence of the protein encoded by ORF-2 exhibited a high level of identity and similarity with other PMM and PGM/PMM proteins from a variety of species, including *Zymomonas mobilis* (57%/73%, identity/similarity) (AAD5692), PMM from *Sphingomonas S7* (62%/75%) (*14*), PGM/PMM from *S. paucimobilis* ATCC 31461 (62%/75%) (*3*) and PMM/PGM from *Pseudomonas aeruginosa* (39%/55%) (*15*). Analysis of the deduced amino acid sequence of 479 residues also revealed three conserved domains of the phosphohexomutase family (Fig. 1); however, the sequence in the sugar-binding loop (GEM) was found in the PMM/PGM subclass. Therefore, it is highly likely that ORF-2 encodes the bifunctional protein with both PGM and PMM activities.

Expression and Purification—To express ORF-1 and ORF-2, the ORFs were inserted into the expression vector pET-15, yielding the new constructs pSP-1 and pSP-2, respectively. Proteins designated as SP-1 and SP-2 corresponding to ORF-1 and ORF-2, were expressed as fusion proteins with $(His)_6$-tag at the N-terminus. The *E. coli* BL21 (DE3) cells transformed with pSP-1 or pSP-2 were cultured in LB broth at 37°C. SDS–PAGE analysis of cell crude extracts in each *E. coli* strain upon induction with IPTG revealed a prominent band at the position corresponding to the molecular mass calculated from the deduced amino acid sequences of ORF-1 and ORF-2 plus 6XHis (57.5 and 49.9 kDa, respectively). The proteins in the soluble fraction were purified to homogeneity by affinity chromatography as indicated in Fig. 2. The molecular mass of subunits of SP-1 and SP-2 were estimated at 58 and 51 kDa from SDS–PAGE, respectively. The native molecular masses for SP-1 and SP-2 were determined to be ~59.5 and 105.4 kDa by non-denaturing PAGE, respectively, indicating that SP-1 and SP-2 likely exist as a monomer and a dimer in native condition (Fig. 3) (*16*, *17*). Purified proteins were stored at −20°C in the presence of 5% glycerol and used for subsequent enzymatic analysis.

	Domain I	Domain II	Domain III
	Active Site	Metal Binding Site	Sugar Binding Site
SP-1	LSASHNPG	SDGDGDRN	CGEESA
M.loti PGM	LSASHNPG	SDGDGDRN	CGEESA
Rabbit PGM	LTASHNPG	FDGDGDRN	CGEESF
SP-2	ITGSHNPA	FDGDGDRI	GGEMTG
S.pau PMM/PGM	ITGSHNPG	FDGDGDRL	AGEMSG
P.aur PGM/PMM	LTGSHNPP	FDGDGDRV	AGEMSG

Fig. 1. **Conserved sequences of PGM and the PGM/PMM family.** *M. loti*, *Mesorhizobium loti* (NCBI accession no.: AAK58597); *S. pau, S. paucimobilis* ATCC 31461 (NCBI accession no.: AAF03690); *P. aur*, *Pseudomonas aeruginosa* (NCBI accession no.: P26276); *Rabbit PGM* (NCBI accession no.: 1C4G_B).

Enzyme Activity—Both SP-1 and SP-2 proteins showed PMM as well as PGM activities detected by an increase in absorbance at 340 nm over various time periods (data not shown). Two proteins can utilize a variety of metal ions to activate PGM activity (Fig. 4). No PGM activity in SP-1 or SP-2 was observed when the divalent metal ions were removed from the assay buffer by addition of 0.1 mM EDTA, indicating that the PGM activity of SP-1 and SP-2 requires the divalent metal ions. The activation efficiency by all tested metal ions seemed similar in the PGM activity of SP-1, whereas the Mg^{2+} ion gave the highest PGM activity for SP-2. The proteins SP-1 and SP-2 exhibited high PGM and PMM activities between pH 7.0 and 9.0 with maximal activity around pH 7.5 (Fig. 5).

Kinetic Characterization of SP-1 and SP-2 Proteins—Plots of initial velocity versus substrate concentration were made to determine the effect of substrate concentration on enzyme activity. The reaction conditions for assay were a pH of 7.5 and substrate concentrations of both M1P and G1P over the range of 0.125–3.0 mM. Kinetic analysis of SP-1 and SP-2 revealed that the dependence of PGM and PMM activity on substrate concentration showed a typical Michaelis–Menten saturation pattern (Fig. 6). The apparent K_m and V_{max}

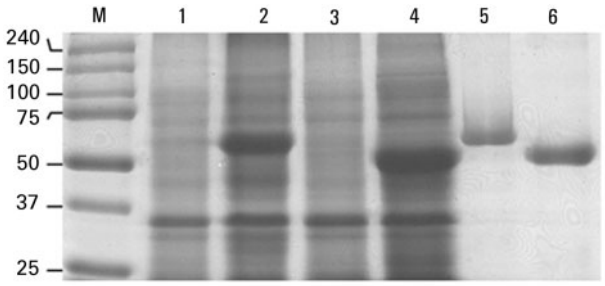

Fig. 2. **SDS–PAGE analysis of protein expression and the purified proteins.** Lanes: M, molecular markers in kDa; 1, non-induced cell crude total extract of pSP-1; 2, induced cell crude total extract of pSP-1; 3, non-induced cell crude total extract of pSP-2; 4, induced cell crude total extract of pSP-2; 5, purified SP-1 protein; 6, purified SP-2 protein.

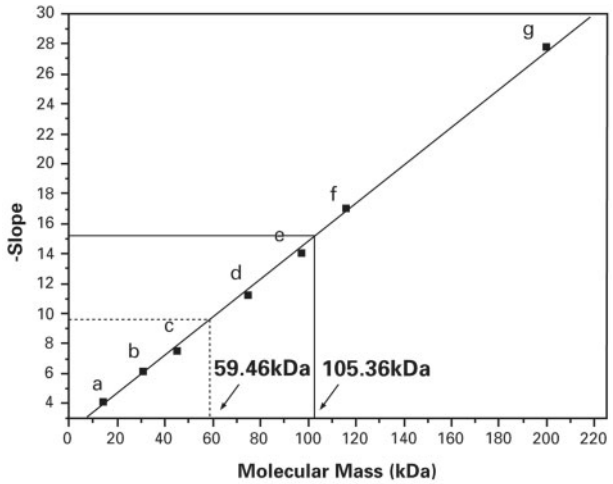

Fig. 3. **Molecular mass determination of the active forms of SP-1 (dotted line) and SP-2 (solid line) by non-denaturing gel electrophoresis.** The estimation methods are followed by Hedrick and Smith's one with a slight modification (*17*). Graph of the slope of $100 \times \log (R_f \times 100)$ versus gel concentration (%) as a function of the molecular mass. The slopes were obtained for seven proteins. a, lysozyme; b, carbonic anhydrase; c, ovalbumin; d, bovine serum albumin; e, phosphorylase b; f, β-galactosidase; g, myosin.

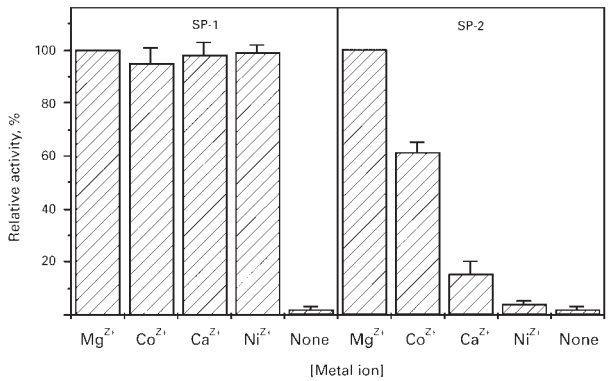

Fig. 4. **PGM activities of SP-1 and SP-2 in the presence of different metal ions.** The relative activity is shown as percentage of activity determined in the presence of Mg^{2+}. Metal ion concentration was 2 mM.

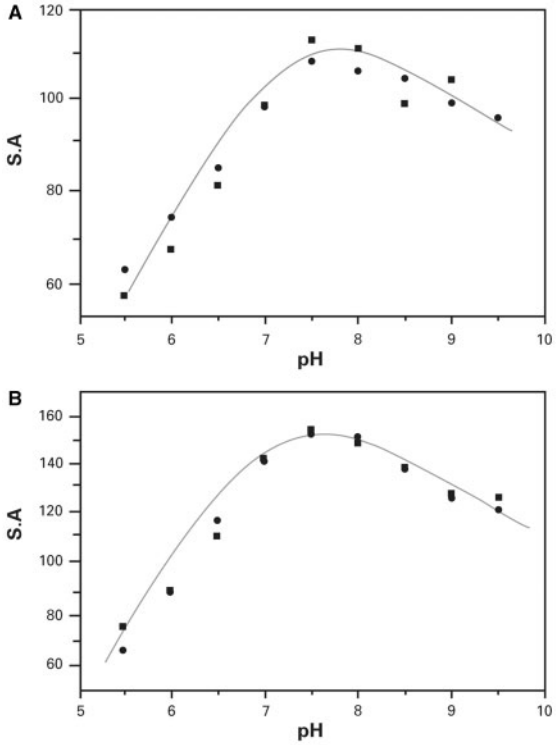

Fig. 5. **The pH-dependent specific activities (S.A., μmol/min/mg) of SP-1 for PGM activity (A), SP-2 for PMM activity (B).** Activity was measured at 2.0 mM G1P/M1P. The pH of assay solution was adjusted using MES (5.5–6.5), MOPS (6.5–8.0) and TAPS (8.0–9.5) buffers.

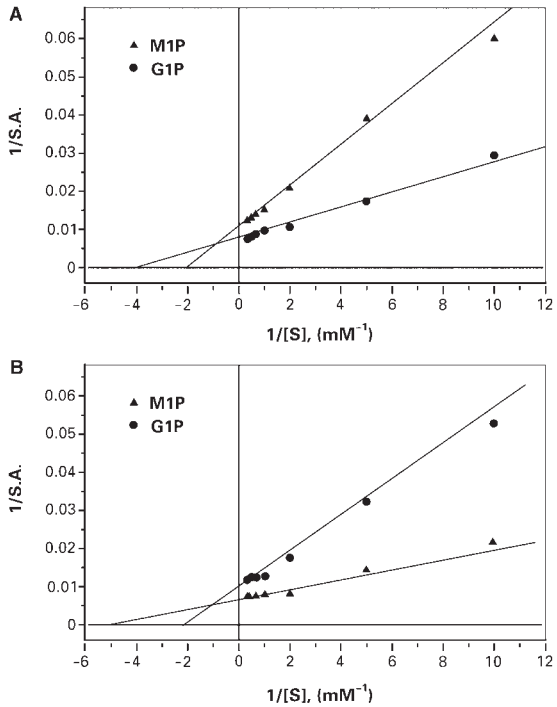

Fig. 6. **The Lineweaver–Burk plots for the reaction rates of SP-1 and SP-2 depending on substrate property and concentration.** (A) Specific activities (S.A., μmol/min/mg) of SP-1 for G1P and M1P. (B) Specific activities of SP-2 for G1P and M1P.

Table 2. **Kinetic properties of SP-1 and SP-2.**

Proteins	SP-1		SP-2	
Substrates	G1P	M1P	G1P	M1P
K_m (μM)	222.8	439.2	451.6	156.9
V_{max} (μmol/min/mg)	132.1	82.5	99.6	162.0
k_{cat} (min^{-1})	7,609.0	4,750.3	4,976.0	8,096.8
k_{cat}/K_m (min^{-1}/μM)	33.2	10.8	11.0	51.6

of both SP-1 and SP-2 for G1P and M1P are listed in Table 2. The SP-1 enzyme protein showed a preference to G1P, as indicated by the twice-lower K_m for G1P than for M1P. The catalytic efficiency (k_{cat}/K_m) of SP-1 for G1P was also ~3-fold higher than that for M1P. In contrast to SP-1, M1P seemed to be a favourable substrate for SP-2 because the K_m for M1P was 3-fold lower than that for G1P and the catalytic efficiency of SP-2 for M1P was 5-fold higher than that for G1P.

DISCUSSION

In this study, the genes encoding proteins that have both PGM and PMM activities were first cloned in *S. chungbukensis* DJ77. Their deduced amino acid sequences contain three highly conserved domains of the phosphohexomutase family including an active site and metal- and sugar-binding domains.

The SP-1 sequence was highly homologous with other members of the PGM subgroup and contained class-specific residues in the substrate binding loop of PGM subgroup (GEES) (*14*) exhibiting only PGM activity. Furthermore, SP-1 can efficiently catalyse the interconversion of G1P to G6P in the presence of metal ions. This phenomenon has also been reported for rabbit PGM (*7*). Ni^{2+} and Co^{2+} can activate rabbit PGM but not *Pseudomonas* PMM/PGM (*7*). These observations suggest that SP-1 likely contains only PGM activity; however, activity assay indicated that this protein possesses both PGM and PMM, and that the catalytic efficiency was higher for G1P. The K_m value for G1P 223 μM was similar to that of PGM/PMM from *S. paucimobilis* ATCC 31461 (330 μM) (*3*) and much higher than that of rabbit PGM (8 μM) (*18*).

A comparison of the SP-2 amino acid sequence with PMM or PGM/PMM found in the database revealed a high level of similarity with PGM/PMM and PMM proteins from other sources. The class-specific residues in the sugar-binding loop of the PGM/PMM subclass (GEMS) were also found in the SP-2 sequence. It was reported that the well-known *Pseudomonas* PGM/PMM requires Mg^{2+} for maximal PGM activity and that neither Co^{2+} nor Ca^{2+} function as a co-factor (*10*). In this study, SP-2 exhibited PGM activity even in the presence of Co^{2+} or Ca^{2+}. The utilization of variety of metal ions by PGM/PMM was also reported for the hyperthermophilic archaeon *Pyrococcus horikoshii* (*19*). SP-2 showed a preference for PMM activity, while other identified proteins in the PGM/PMM subgroup were more favourable towards PGM activity. This phenomenon might be explained by the presence of an SP-1 specific to phosphoglucose isomerization in *S. chungbukensis* DJ77.

The K_m of both SP-1 and SP-2 for G1P or M1P was relatively high, indicating that the reverse reactions from G6P or M6P to G1P or M1P, respectively, are favourable. Therefore, metabolic flux was tilted towards polymer synthesis rather than a catabolic pathway. The next step of our investigations will be to characterize EPS synthesis upon disruption of either one or both genes in *S. chungbukensis* DJ77 and thereby evaluate the contribution of these proteins to EPS synthesis.

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CONFLICT OF INTEREST

None declared.

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