The α-Phosphoglucomutase of Lactococcus lactis Is Unrelated to the α-Δ-Phosphohexomutase Superfamily and Is Encoded by the Essential Gene pgmH

Ana R. Neves1, Wietske A. Pool9, Rute Castro4, Ana Mingote9, Filipe Santos5, Jan Kok1, Oscar P. Kuipers5, and Helena Santos12

From the 1Instituto de Tecnologia Química e Biomolecular and Instituto de Biologia Experimental e Tecnológica, Universidade Nova de Lisboa, Rua da Quinta Grande, 6, Apartado 127, 2780-156 Oeiras, Portugal and the 4Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9750 AA Haren, The Netherlands

α-Phosphoglucomutase (α-PGM) plays an important role in carbohydrate metabolism by catalyzing the reversible conversion of α-glucose 1-phosphate to glucose 6-phosphate. Isolation of α-PGM activity from cell extracts of Lactococcus lactis strain MG1363 led to the conclusion that this activity is encoded by yfgH, herein renamed pgmH. Its gene product has no sequence homology to proteins in the α-Δ-phosphohexomutase superfamily and is instead related to the eukaryotic phosphomannomutases within the haloacid dehalogenase superfamily. In contrast to known bacterial α-PGMs, this 28-kDa enzyme is highly specific for α-glucose 1-phosphate and glucose 6-phosphate and showed no activity for mannose phosphate. To elucidate the function of pgmH, the metabolism of glucose and galactose was characterized in mutants overproducing or with a deficiency of α-PGM activity. Overproduction of α-PGM led to increased glycolytic flux and growth rate on galactose. Despite several attempts, we failed to obtain a deletion mutant of pgmH. The essentiality of this gene was proven by using a conditional knock-out strain in which a native copy of the gene was provided in trans under the control of the nisin promoter. Growth of this strain was severely impaired when α-PGM activity was below the control level. We show that the novel L. lactis α-PGM is the only enzyme that mediates the interconversion of α-glucose 1-phosphate to glucose 6-phosphate and is essential for growth.

Phosphoglucomutase (PGM; EC 5.4.2.2) is widespread in living organisms from bacteria to humans (1). It plays various roles in carbohydrate metabolism by catalyzing the reversible conversion of α-Glc-1-P to Glc-6-P. In higher organisms, its major function is mediating the mobilization of sugar moieties from energy reserves. Also, α-PGM activity is essential for the synthesis of UDP-glucose, a sugar donor for the production of glucose-containing polysaccharides. Therefore, PGM is a crucial link between catabolic and anabolic processes.

Lactococcus lactis is used worldwide in the industrial manufacture of fermented milk products. The organism converts sugars primarily into lactic acid, thus providing an efficient means of food conservation. In L. lactis, PGM is assumed to be essential for the utilization of galactose via the Leloir pathway (2) and for the synthesis of cell wall polysaccharides and exopolysaccharides (3, 4). In a number of Gram-positive bacteria, pgm mutants show altered cell wall morphology and altered polysaccharide production as well as growth defects on glucose (5–7). Despite the wealth of knowledge on sugar metabolism of L. lactis (8), genes coding for α-PGM have not been identified in this organism.

More than 1 decade ago, the presence of two distinct PGM activities in L. lactis ssp. lactis with specificity for α- and β-anomers of phosphoglucone was reported (9). A 28-kDa protein (designated β-PGM) was shown to catalyze the reversible conversion of β-Glc-1-P to Glc-6-P. L. lactis β-PGM, which belongs to the haloacid dehalogenase (HAD) superfamily (10–12), is a catabolic enzyme in the pathway for maltose and trehalose degradation encoded by a gene (pgmB) in the trehalose operon (13–15). A larger protein (∼65 kDa) showing affinity for the α-anomer of Glc-1-P was partially purified (9). The α-specificity and protein size, which matched the analogous parameters of bacterial α-PGMs, led to the conclusion that it was the lactococcal α-PGM. Thus far, all α-PGMs described belong to the α-Δ-phosphohexomutase superfamily of proteins (1, 16). Intriguingly, a BLASTp search of the available L. lactis genome sequences (14) using members of the α-Δ-phosphohexomutase superfamily as query or a domain search using the highly conserved regions of proteins in this family retrieved only FemD, a protein tentatively annotated as a phosphogluco-amine mutase.

In this work, we report the identification, purification, expression, and characterization of L. lactis α-PGM and its
encoding gene pgmH (previously known as yfgH) and show that this activity is essential for growth. To our knowledge, lactococcal α-PGM is the first member of the HAD superfamily of proteins with strict specificity for α-Glc-1-P.

**EXPERIMENTAL PROCEDURES**

**Microbial Strains and Growth Conditions**—The strains and plasmids used throughout this study are listed in Table 1. For general molecular biology procedures, *L. lactis* strains were cultivated as batch cultures (flasks) without aeration in M17 medium (Difco) containing 0.5% (w/v) glucose at 30 or 38 °C. For physiological studies, *L. lactis* NZ9000 (17) derivatives NZ9000 (pNZ8048) (control strain), NZ9000 (pNZ8048-pgmH) (hereafter designated NZ9000 [pgmH⁺]), and NZ9000 ΔpgmH (pgmH⁻) were grown in chemically defined medium used at 30 °C under anaerobic conditions at pH 6.5 as described previously (18). Glucose or galactose was added to a final concentration of 1% (w/v). Plasmid selection was achieved by the addition of chloramphenicol (5 mg/liter) or erythromycin (5 mg/liter). For overproduction of α-PGM, nisin (1 μg/liter) was added at A₆₀₀ = 0.5. For studies in which the nisin-inducible conditional mutant NZ9000 ΔpgmH/pgmH⁺ was used, cells were grown in M17 medium containing different levels of nisin and washed once with fresh M17 medium lacking nisin. The cultures were subsequently subcultured for 15 h in fresh medium with or without nisin (0.01–1 μg/liter).

**DNA Techniques**—General molecular techniques were performed as described by Sambrook et al. (19). Chromosomal and plasmid DNAs were isolated from *L. lactis* according to Johansen and Kibenich (20) and Birnboim (21), respectively. *L. lactis* was transformed with plasmid DNA by electroporation as described by Holo and Nes (22). All DNA modification enzymes were purchased from Roche Applied Science (Mannheim, Germany). PCRs were performed using Expand DNA polymerase and purified with a Roche PCR purification kit. Primers (listed in supplemental Table 1SM) were purchased from Biologio BV (Malden, The Netherlands).

**Construction of Strains and Plasmids**—The genes pgmH and galE were cloned and overexpressed in *L. lactis* strain NZ9000 as follows. The coding regions of pgmH and galE were amplified by PCR using primer pairs yfgH-fw/yfgH-rev and galE-fw/galE-rev. The 0.76-kb Rcal/Xbal and 0.98-kb Rcal/Spel fragments were digested with the indicated enzymes and cloned into NcoI/Xbal- or NcoI/SpeI-digested pNZ8048 (17), yielding constructs pNZ8048-pgmH and pNZ8048-galE, respectively. The femD gene was amplified using primer pair femD-fw/femD-rev. The pNZ8048-femD overexpression plasmid was constructed by cloning the 1.36-kb NcoI/XbaI PCR product into similarly digested pNZ8048. The resulting construct was transformed into *L. lactis* strain NZ9000. *L. lactis* MG1363 DNA (23) was used as template for all PCRs.

Several strategies were employed to construct an *L. lactis* ΔpgmH strain. A complete deletion of the pgmH gene was tried by using a two-step homologous recombination method as follows. The upstream and downstream flanking regions of pgmH were obtained by PCR using primer pairs yfgH-KO1/yfgH-KO2 and yfgH-KO3/yfgH-KO4 and cloned as EcoRI/BamHI and BamHI/XbaI restriction fragments into pORI1280 (24), resulting in plasmid pORI1280ΔpgmH. The plasmid was obtained and maintained in *L. lactis* LL302 (25). pORI1280ΔpgmH and pVE6007 (26) were cotransformed into NZ9000, and this strain was taken through the temperature shift protocol for single and double crossovers (24). No double crossover transformants were obtained in M17 medium supplemented with folowing sugars at 0.5%: glucose, maltose, and trehalose or a mixture of trehalose (0.5%) and galactose (0.05%). In a second approach, a 0.46-bp fragment containing the 5’-end of pgmH except for 2 bp was amplified by PCR using primers yfgH-fw1 and yfgH-rev1, double-digested with EcoRI/XbaI, and cloned into similarly digested pORI13 (27). The resulting plasmid was transformed into NZ9000, but despite several attempts, no erythromycin-resistant colonies were obtained.

Subsequently, pNZ8048-pgmH was introduced in NZ9000::pORI1280ΔpgmH, obtained as explained above. The resulting chloramphenicol- and erythromycin-resistant strain was subjected to an excision strategy (24) in M17 medium containing glucose and nisin (0.1 μg/liter), yielding NZ9000 ΔpgmH/pgmH⁺. The addition of nisin was required to induce expression of pgmH from pNZ8048-pgmH.

Integration of plasmids in the chromosome and deletions were confirmed by PCR and Southern blotting. Probe labeling,
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hybridization, and detection were performed using the ECL direct nucleic acid labeling system (Amersham Biosciences, Little Chalfont, UK).

**Enzyme Assays**—Enzymes were assayed at 30 °C after disruption of a cell suspension by passage through a French press (twice at 120 megapascals). Protein concentration was determined by the method of Bradford (28). Routinely and during enzyme purification, α- and β-PGM activities were assayed as described by Qian et al. (9). Reactions were started by the addition of 1.5 mM α-Glc-1-P or β-Glc-1-P, respectively.

**Purification of Native α-PGM from L. lactis**—All steps were performed in the presence of 0.5 mM EDTA and 5 mM 2-mercaptoethanol at 4 °C. Cell extracts were prepared from 135 g (wet weight) of galactose-grown MG1363 cells that were suspended in 50 mM triethanolamine (TEA) buffer (pH 7.2) containing 5 mM MgCl₂. Precipitation steps with protamine sulfate (0.25%, w/v) and solid ammonium sulfate were performed as described by Qian et al. (9). The precipitate in the range of 45–85% (NH₄)₂SO₄ saturation was dissolved in 50 mM TEA buffer (pH 7.2) containing 30 mM KCl and dialyzed against the same buffer. The sample was applied to a Superdex 200 gel filtration column in 20 mM BisTris propane (pH 6.9) containing 45 mM KCl, and α-PGM activity was detected in the flow-through fractions. Active samples were loaded onto a Resource Q column in the same buffer, and elution was carried out with a linear gradient of KCl (45–500 mM). Active fractions (which eluted at ~240 mM KCl) were dialyzed against 10 mM Tris-HCl (pH 7.3) containing 4 mM MgCl₂ and 1.6 M (NH₄)₂SO₄. The positive fractions were dialyzed against 50 mM TEA buffer (pH 7.5) and 30 mM KCl and loaded onto a Superose 6 gel filtration column. α-PGM activity was measured in the flow-through fractions. Two putative target proteins of 28 and 37 kDa were excised from Coomassie Blue-stained SDS-polyacrylamide gels, and the amino acid sequences of their N termini were determined (29). Open reading frames encoding the two proteins were identified by BLASTp searches using the genome sequence of *L. lactis* ssp. *lactis* IL1403.

**Purification and Characterization of Recombinant α-PGM**—The protein was purified to electrophoretic homogeneity from ~65 g (wet weight) of glucose-grown nisin-induced NZ9000(pgmH') cells. A purification strategy similar to that used for the native enzyme was followed, except that the Superdex 200 gel filtration column was replaced with an anion-exchange Q-Sepharose column in 20 mM BisTris propane (pH 6.9) with a 45–500 mM KCl gradient, and the last chromatographic step (gel filtration) was not required. Also, the hydrophobic step (phenyl-Sepharose) preceded the anion-exchange step (Resource Q). The purified protein was stored at −20 °C in TEA buffer.

The pH profile was determined in TEA buffer at a range of 4–9. The effect of alternative cations (Ni²⁺, Zn²⁺, Ca²⁺, Mn²⁺, and Li⁺) at 1 or 5 mM was examined in the presence of 50 μM Mg²⁺. ATP and fructose 1,6-bisphosphate (Fru-1,6-P₂) were examined as potential inhibitors of α-PGM. Kinetic constants were determined in the reaction direction α-Glc-1-P → Glc-6-P. The *V*ₘₐₓ for the direction Glc-6-P → α-Glc-1-P and substrate specificity were determined by ³¹P NMR spectroscopy. The 3-mi reaction mixtures contained 50 mM TEA buffer (pH 7.2), 5 mM MgCl₂, 50 mM Glc-1,6-P₂, 3% (v/v) H₂O, and 20 μg of pure enzyme. For substrate specificity, spectra were acquired before and after 3 h of incubation at 30 °C in the presence of the following compounds at 7.5 mM: α-Glc-1-P, Glc-6-P, β-Glc-1-P, Glcn-1-P, Glcn-6-P, α-Man-1-P, Fru-1-P, Fru-6-P, Fru-1,6-P₂, Fru-1,6-P₂/α-Glc-1-P, α-Gal-1-P, Gal-6-P, Rib-5-P, ribulose-5-P, 6-phosphogluconate, UDP-Gal, and UDP-Glc.

For *V*ₘₐₓ determinations, the reactions were started by the addition of Glc-6-P (50 μM), and the time course for its consumption was monitored. Methyl phosphonate was added as an internal concentration standard. Molecular mass was determined by gel filtration on a Superose 12 10/300 GL column using 100 mM sodium acetate (pH 7.0).

**Determination of Extracellular Metabolites during Growth**—Samples (2 ml) of NZ9000(pNZ8048) or NZ9000-(pgmH') cultures growing in chemically defined medium containing either glucose or galactose were collected at different points during growth. Fermentation substrates and products were quantified as described (30).

Ethanol extracts for analysis by ³¹P NMR and quantification of phosphorylated metabolites in NZ9000(pgmH') and control strains at mid-exponential growth phase were prepared as described (31). Assignment of resonances and quantification of phosphorylated metabolites were based on previous studies (31) or determined by adding the suspected, pure compounds to the NMR sample extracts. The values reported for intracellular phosphorylated compounds are the averages of two independent growth experiments, and the accuracy was ~15%.

**NMR Experiments and Quantification of Metabolites**—Cells were harvested during the mid-logarithmic growth phase (A₆₀₀ = 2.2), centrifuged, washed twice, and resuspended to a protein concentration of 16.5 mg/ml in 50 mM potassium Pi buffer (pH 6.5). In vivo NMR experiments were performed as described (18). Spectra were acquired sequentially prior to and after the addition of [1-¹³C]glucose or [1-¹³C]galactose. After substrate exhaustion and when no changes in the resonances due to end products and intracellular metabolites were observed, a total NMR sample extract was prepared and used for quantification of end products and other metabolites (32–34). Correction factors to convert peak intensities into concentrations for Gal-1-P (0.73), α-Glc-1-P (0.73), UDP-Gal (0.67), and UDP-Glc (0.67) were determined using NMR sample extracts supplemented with the pure compounds as described (32).

**NMR Spectroscopy**—NMR spectra of living cells were run at 30 °C as described by Neves et al. (18). Although individual experiments are depicted in the figures, each experiment was repeated at least twice, and the results were highly reproducible. The concentration values are the means of two to four experiments, and the accuracy varied from ±2% in the case of extracellular products to ±10% in the case of intracellular metabolites with concentrations below 5 mM. The quantification of phosphorylated metabolites and the measurement of α-PGM activity were performed as described by Ramos et al. (31). Carbon and phosphorus chemical shifts are referenced to external methanol or H₃PO₄ (85%) designated at 49.3 and 0.0 ppm, respectively.
Chemicals—[1-13C]Glucose (99% enrichment) and [L-13C]-galactose (99% enrichment) were obtained from Campro Scientific (Veenendaal, The Netherlands) and Cambridge Isotope Laboratories (Andover, MA), respectively. All other chemicals were reagent grade and obtained from Sigma.

RESULTS
Purification of α-PGM Activity and Identification of the Coding Gene

A BLASTp search of L. lactis MG1363 and IL1403 genomes using sequences of proteins in the α-1-phosphohexomutase superfamily identified femD as the best hit. FemD, annotated as a putative phosphoglucosamine mutase (14), is a protein with a calculated molecular mass of 48 kDa. To ascertain whether femD encodes a protein with α-PGM activity, the gene (1356 bp) was cloned into pNZ8048 under the control of the nisin-inducible promoter and introduced into NZ9000. Extracts of nisin-induced NZ9000(pNZ8048-femD) cells contained a clearly overproduced protein with a size of 48 kDa (data not shown). Moreover, the activity of α-PGM in the induced strain was identical to that in control cells (0.15 units/mg of protein), indicating that femD does not encode an α-PGM. A PCR strategy to clone the lactococcal α-PGM gene using degenerate primers based on highly conserved regions of phosphohexomutases (16) and the complementation of an Escherichia coli pgm::tet mutant (35) with a genomic library of L. lactis were also attempted without success. Consequently, purification of α-PGM activity was pursued to obtain protein sequence information and to identify the gene.

α-PGM was partially purified (100-fold). Fractions from the last column contained several bands as visualized by SDS-PAGE, but only two proteins (28 and 37 kDa) co-eluted with the last column contained several bands as visualized by SDS-PAGE, but only two proteins (28 and 37 kDa) co-eluted with the

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The protein was purified ~13-fold to a specific activity of 65 units/mg of protein. The biochemical and kinetic properties are listed in Table 2. The purified protein was electrophoretically homogeneous, and SDS-PAGE revealed a single subunit with an apparent molecular mass of 28 kDa. A mass of 84.3 kDa was determined by gel filtration (Superase 12 10/300 GL), suggesting a trimeric structure. The pH for maximal activity was 6.5; 50% of the activity was found at pH 5.6 and 7.5. The rate dependence on α-Glc-1-P concentration followed Michaelis-Menten kinetics. Several phosphosugars were examined as putative substrates using the 31P NMR direct assay. The enzyme catalyzed only the interconversion of α-Glc-1-P to Glc-6-P. Furthermore, the apparent Vmax value for the reverse direction (Glc-6-P → α-Glc-1-P) was 3-fold lower than that for the forward direction, and the apparent Km value for Glc-6-P was in the millimolar range. Neither α-Man-1-P nor β-Glc-1-P was a substrate for the enzyme, despite the sequence similarity of L. lactis α-PGM to the eukaryotic PMMs (~25%) and β-PGM to Glc-1-P phosphomutases (~10%).

For maximal activity, α-PGM required Mg2+ and Glc-1,6-P2. In the absence of Mg2+, the activity was reduced to <2%, and no activity was detected when Glc-1,6-P2 was omitted from the assay. Zn2+ and Ca2+ strongly inhibited the activity (Table 2). ATP showed no regulatory effect, whereas Fru-1,6-P2 moderately inhibited the activity.

Pools of Glycolytic Intermediates in Non-growing Cells

L. lactis NZ9000 cells harboring pNZ8048-pgmH or pNZ8048 (control) were grown in a chemically defined medium containing glucose or galactose and induced with nisin for α-PGM production. The effect of α-PGM overproduction on the metabolism of glucose and galactose was studied by in vivo 13C NMR (Fig. 2).

[1-13C]Glucose—The conversion of glucose was homofermentative in the NZ9000(pgmH+) and NZ9000(pNZ8048) strains (lactate production above 91%), with the glucose consumption rates being 0.37 ± 0.02 and 0.39 ± 0.02 μmol/min/mg of protein. The dynamics of intracellular metabolite

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pools were not appreciably affected by the substantial increase in \( /H9251\)-PGM activity from 0.07 units/mg of protein in the control to 2.6 units/mg of protein in NZ9000(\(pgmH\)) (Fig. 2, A and B).

The maximal concentration of Fru-1,6-P\(_2\) decreased by 10%, and 3-phosphoglycerate increased slightly. Moreover, the UDP-glucose pool decreased from 6.7 ± 0.5 mM in the control to 4.2 ± 0.3 mM in NZ9000(\(pgmH\)) (Fig. 2, A and B).

[1-\(^{13}\)C]Galactose—As expected for galactose, the metabolism of pyruvate was shifted to products other than lactate, ethanol (2.2 ± 0.2 mM), and acetate (2.4 ± 0.4 mM), but their concentrations were ~35% lower in NZ9000(\(pgmH\)). Induction with nisin resulted in a 7-fold higher (0.3–2.1 units/mg of protein) \( /H9251\)-PGM activity in the strain harboring the \(pgmH\) construct, a modest change compared with the 37-fold increase

**FIGURE 1.** Multiple sequence alignment of amino acid sequences of \(\alpha\)-PGM from \(L.\) lactis ssp. cremoris MG1363 and its putative homologs from \(P.\) acnes, \(B.\) longum, \(M.\) musculus, \(H.\) sapiens (PMM1 and PMM2), and \(S.\) cerevisiae. The alignment was generated with ClustalW. The eukaryotic \(\alpha\)-PMMs belong to subfamily II of the HAD superfamily. Lactococcal \(\beta\)-PGM, a subfamily I member, is shown in gray. The signature pattern for motif I of the HAD superfamily phosphomutases, -DXD- (metal binding and nucleophile), is highlighted by the dark gray box (42). The invariant aspartate residue is shown in yellow. The other signature patterns of HAD superfamily subfamily II (motifs II–IV) are highlighted by the dark green boxes (39, 47). The light blue and light green boxes highlight core motifs 1–4 and cap loop 5, respectively, of \(\beta\)-PGM (43). The yellow boxes indicate hinge regions connecting core and cap domains. Residues proposed to be important for substrate binding or catalysis are shown in red. The conserved serine residue in motif II is shown in green (residue inversion in \(\alpha\)-PMM and lactococcal \(\alpha\)-PGM and its homologs). For accession numbers, see “Results.”
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TABLE 2
Biological and kinetic properties of α-PGM from L. lactis

| Parameter                        | Value  |
|---------------------------------|--------|
| Enzyme apparent molecular mass (kDa) | 84.3   |
| Native                           |        |
| Subunit                         | 28     |
| Calculated                      | 28.3   |
| Oligomeric structure            | α3     |
| pH optimum                      | 6.5 ± 0.5 |
| K_m for α-Glc-1-P (µM)          | 71.4 ± 2.8 |
| K_m for Mg^2+                   | 16.8 ± 1.3 |
| V_max (units/mg protein)        |        |
| α-Glc-1-P                       | 65.3 ± 1.8 |
| α-Glc-6-P                       | 15.8 ± 1.3 |
| Substrate specificity (%)       |        |
| α-Glc-1-P (7.5 mM)              | 100%   |
| α-Glc-6-P (50 mM)               | 23     |
| β-Glc-1-P (7.5 mM)              | 0%     |
| Effect of cations (%)           |        |
| Mg^2+ (0 and 0.05 mM)           | <2' and 49 |
| Zn^2+ (5 and 1 mM)              | 2 and 28 |
| Ca^2+ (5 and 1 mM)              | 0.3 and 1.6 |
| Mn^2+ (5 and 1 mM)              | 17 and 21 |
| Ni^2+ (5 and 1 mM)              | 43 and 41 |
| Li^+ (5 and 1 mM)               | 42 and 39 |
| Potential inhibitors (I_{50} mm) |        |
| Fru-1,6-P1                      | 48 ± 1.1 |
| ATP                             | No effect |

α Activities are relative to the value determined for the conversion of α-Glc-1-P to Glc-6-P (which was set to 100%) as measured by 31P NMR.

The following phosphosugars were also examined, but no activity was detected: Glc-1,6-P2, α-Man-1-P, Fru-1-P, Fru-6-P, Fructose-6-P, Glc-1-P, Glc-6-P, Rib-5-P, ribulose-5-P, 6-phosphogluconate, UDP-Gal, and UDP-Glc. When a mixture of Fru-1,6-P2 and α-Glc-1-P was used, α-Glc-1-P was fully converted to Glc-6-P, but Fru-1,6-P2 was not used.

Activity is relative to the value determined for the conversion of α-Glc-1-P to Glc-6-P (which was set to 100%) as measured using the standard coupling assay and 5 mM Mg^2+.

observed in glucose-grown cells. Curiously, the galactose consumption rate was 25% greater (from 0.16 ± 0.1 to 0.21 ± 0.1 µmol/min/mg of protein) in the strain overproducing α-PGM. Overproduction of α-PGM had a considerable impact on the concentrations of intracellular metabolite pools (Fig. 2, C and D). A remarkable reduction in the size of the Gal-1-P and α-Glc-1-P pools to 2.4 and 2.2 mM, respectively, revealed α-PGM as the main bottleneck during galactose metabolism in L. lactis. UDP-Glc (3.9 ± 0.4 mM) and UDP-Gal (3.2 ± 0.3 mM) were detected, and these pools were 1.4-fold lower in the strain carrying the plasmid pNZ8048, most likely due to low basal P_nisA expression. Galactose per se induced α-PGM activity 2- and 4-fold in NZ9000(pgmH^+) and the control strain, respectively (Table 3). Up-regulation of pgmH expression on galactose was also observed for the parent strains MG1363 and NZ9000.

The growth rate was affected by nisin addition, but the magnitude and sign of the effect were sugar-dependent (Table 3). The growth rate on glucose decreased considerably upon the addition of nisin, but this negative effect was unrelated to α-PGM overproduction (16-fold increase). Despite the lower α-PGM activity achieved on galactose (7-fold increase), the growth rate of NZ9000(pgmH^+) was substantially greater than that of the control strain (0.50 vs 0.36 h^-1), reaching a value close to that on glucose (0.56 h^-1). The results show that α-PGM activity in the control strain was limiting during growth on galactose and that this bottleneck was overcome by pgmH overexpression.

In glucose-grown cells, increased α-PGM activity had no impact on product formation, with lactate accounting for >90% of the end products. When galactose was used as the carbon source, the lactate yield increased slightly in the strain overproducing α-PGM (Fig. 3 and Table 3), in line with the increased growth rate of NZ9000(pgmH^+). Biomass yield, ATP yield, and biomass yield on ATP were dependent on the sugar used and were not affected by overproduction of α-PGM (Table 3).

Pools of Phosphorylated Metabolites—Pool sizes for glycolytic intermediates and sugar nucleotides were determined by 31P NMR in cell extracts derived from mid-exponential phase cultures (supplemental Table SM2). Fru-1,6-P2 was the major metabolite on glucose, whereas galactose 3-phosphoglycerate and phosphoenolpyruvate were also present in high amounts. As expected, the Leloir pathway intermediate Gal-1-P was below the detection limit on glucose; on galactose, its concentration was slightly lower (20%) in the α-PGM-overproducing strain. The concentration of α-Glc-1-P decreased notably in NZ9000(pgmH^+) regardless of the sugar used.

It is thought that, in L. lactis, UDP-sugars and UDP-amino-sugars are derived from α-Glc-1-P and Fru-6-P, respectively; hence, their concentrations could respond to changes in α-PGM activity. Overproduction of α-PGM resulted in reduction or constancy of UDP-sugars on galactose- or glucose-grown cells, respectively. Concentrations of UDP-amino-sugars were not significantly affected by pgmH overexpression, except for UDP-N-acetylglucosamine, the first cytoplasmic precursor of peptidoglycan, the level of which responded inversely to an increase in the activity of α-PGM.

Inactivation of the Chromosomal pgmH Gene and Its Effect on L. lactis Growth

To investigate whether α-PGM activity is essential for growth of L. lactis on glucose and galactose, we decided to inactivate the pgmH gene. Several attempts to disrupt pgmH by single crossover plasmid integration with pORI13-pgmH^+ or by a method to introduce unmarked deletions failed. These results suggest that pgmH plays an essential role in L. lactis. Integration of pOR1280ΔpgmH in NZ9000 resulted in erythromycin-resistant colonies harboring a disrupted as well as an integral copy of pgmH. Only when pgmH was expressed in trans (under nisin
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control in pNZ8048) in NZ9000::pORI280ΔpgmH was it possible to delete the chromosomal copy of pgmH, as confirmed by PCR and Southern analysis (data not shown). The resulting strain, NZ9000ΔpgmH(pgmH^+) was constructed and maintained in the presence of 0.1 μg/liter nisin. To ascertain whether α-PGM was limiting during growth on Glc-containing M17 medium, low nisin (0.01 μg/liter)-grown cultures (α-PGM activity of 0.11 units/mg of protein) were subcultured in fresh medium with increasing concentrations of nisin (0–1 μg/liter). In the absence of nisin, the mutant strain showed poor growth, with an average growth constant of 0.20 h⁻¹; at 12 h, α-PGM activity had decreased to 0.025 units/mg of protein. Under the same conditions, the NZ9000(pNZ8048) control strain showed a growth rate of 0.55 h⁻¹ and reached a 5-fold higher biomass concentration (A₆₀₀ = 3.2 compared with 0.6) and a steady α-PGM activity of 0.15 units/mg of protein. Modulation of pgmH expression by varying the nisin concentration from 0.01 to 1 μg/liter resulted in a series of cultures with α-PGM activity between 63 and 4000% of the control level (supplemental Fig. 2SM). The maximal growth rate was negatively affected at the lowest α-PGM activity (63% of the control). The data show that α-PGM activities below the control level did not sustain maximal growth of *L. lactis* on Glc-containing M17 medium.

The effect of controlled limitation of pgmH expression was evaluated during growth on glucose and galactose in M17 medium. Strain NZ9000ΔpgmH(pgmH^+) was grown in medium containing different concentrations of nisin and subsequently subcultured in nisin-free medium. In Glc-grown cells, nisin concentrations of 0.1, 0.01, and 0 μg/liter in the inocula resulted in 16, 65, and 74% reductions in the growth rates, respectively. In contrast, nisin concentrations of 0.5 and 1 μg/liter supported growth constants similar to those of the control strain. The effect on growth rates, as well as the stepwise reduction in the final absorbances, correlated well with the decline of α-PGM activity (supplemental Fig. 2SM). These results show that pgmH is essential for growth of *L. lactis* on glucose.

When galactose was used as the sole carbon source, the effect of limiting the expression of pgmH on the growth rates and final absorbance was more pronounced than with glucose. Regardless of the nisin concentration used (0–1 μg/liter), the

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**TABLE 3**

Effect of pgmH overexpression on some growth properties during glucose or galactose fermentation by *L. lactis*

NZ9000(pgmH^+) and NZ9000(pNZ8048) were grown in chemically defined medium supplemented with chloramphenicol (5 mg/liter) and 1% (w/v) glucose or galactose. Nisin (1 μg/liter) was added when the culture reached A₆₀₀ = 0.5. Growth rate constants for the entire growth phase were calculated using linear regressions. α-PGM activity was measured before (A₆₀₀ = 0.5) and after (A₆₀₀ = 2.2) induction. Y_ATP is the biomass yield relative to ATP production. The global ATP yields were calculated from the fermentation products assuming that all ATP was synthesized by substrate level phosphorylation.

| Glucose | pNZ8048 | pgmH^+ |
|---------|---------|--------|
| Carbon balance (%) | 95 ± 0.5 | 95 ± 0.5 |
| Biomass yield (g/mol) | 29.9 ± 0.3 | 29.1 ± 0.3 |
| ATP yield (mol/mol substrate) | 1.9 ± 0.01 | 1.9 ± 0.01 |
| Y_ATP | 15.6 ± 0.02 | 15.2 ± 0.02 |
| μ| 0.80 ± 0.01 | 0.82 ± 0.01 |
| μm | 0.58 ± 0.01 | 0.56 ± 0.02 |
| α-PGM activity (units/mg)^a | 0.07 ± 0.01 | 0.16 ± 0.01 |
| α-PGM activity (units/mg)^b | 0.07 ± 0.01 | 2.63 ± 0.20 |
| Lactate/substrate (%) | 92 ± 4 | 91 ± 4 |
| Other products/substrate (%) | 2 ± 0.5 | 3 ± 0.7 |

| Galactose | pNZ8048 | pgmH^+ |
|----------|---------|--------|
| Carbon balance (%) | 94 ± 0.3 | 94 ± 0.1 |
| Biomass yield (g/mol) | 26.5 ± 0.2 | 26.5 ± 0.4 |
| ATP yield (mol/mol substrate) | 2.1 ± 0.01 | 2.1 ± 0.01 |
| Y_ATP | 12.5 ± 0.02 | 12.9 ± 0.02 |
| μ| 0.36 ± 0.01 | 0.45 ± 0.01 |
| μm | 0.37 ± 0.01 | 0.50 ± 0.01 |
| α-PGM activity (units/mg)^a | 0.29 ± 0.01 | 0.36 ± 0.02 |
| α-PGM activity (units/mg)^b | 0.34 ± 0.02 | 2.05 ± 0.16 |
| Lactate/substrate (%) | 71 ± 1.0 | 79 ± 0.8 |
| Other products/substrate (%) | 23 ± 0.3 | 16 ± 0.3 |

^a Before the addition of nisin.

^b After the addition of nisin (1 μg/liter).
final biomass and growth rate constants were below 60% of the respective parameters in the control strain (\(A_{600} = 3.5\) and \(\mu = 0.41\) h\(^{-1}\)). An \(\alpha\)-PGM activity of 1.38 units/mg of protein was measured at 12 h in cells grown and subcultured in fresh medium with nisin at 0.5 \(\mu\)g/liter, but the growth parameters did not improve. Although no final explanation for this intriguing behavior can be put forward, it is possible that complex regulatory mechanisms resulting from cross-reactions involving galactose metabolism and nisin induction are implicated (40, 41). The severe growth defects shown here for \(L.\) lactis NZ9000 \(\Delta pgmH\) validate the conclusion that \(pgmH\) is essential for growth of \(L.\) lactis on galactose.

**FIGURE 3.** Unrooted phylogenetic tree based on available amino acid sequences of phosphomutases. The ClustalX program (48) was used for sequence alignments and to generate the phylogenetic tree with the neighbor-joining tree construction method. The significance of the branching order was evaluated by bootstrap analysis of 1000 computer-generated trees. The bootstrap values are indicated. Bar = 0.1 change/site. The species and GenBank\textsuperscript{TM} accession numbers are as follows: Oryctolagus cuniculus (Ocun) PGM (P00949); S. cerevisiae (Scer) PGM (NP_013823) and PMM (NP_116609); H. sapiens (Hsap) PGM (AAH67763), phosphoacetylglucosamine mutase (PAGM; NP_013823), \(\beta\)-PGM (PMM; NP_266585), and YfA (NP_266899); Streptococcus pneumoniae (Spen) phosphoacetylglucosamine mutase (PAGM; AAL00221) and PMM/PGM (AAD56627); Streptococcus pyogenes (Spyo) phosphoglucosamine mutase (YP_280210), PMM/PGM (YP_282301), and \(\beta\)-PGM (YP_281891); Bacillus subtilis (Bsub) phosphoglucosamine mutase (NP_388058), PMM/PGM (CAH04980), and \(\beta\)-PGM (NP_391335); E. coli (Ecol) phosphoglucosamine mutase (AAC76208), PMM (O85343), and PGM (AAC73782); M. musculus (Mmus) phosphoglucosamine mutase (NP_082628) and PMM (NP_058577); Drosophila melanogaster (Dmel) phosphoacetylglucosamine mutase (NP_648588); P. acnes (Pacn) hypothetical protein (HP; YP_056695); B. longum (Blon) hypothetical protein (ZP_00121741) and PG (NP_696782); G. zeae (Gzoe) PMM (EAA17459); Oryza sativa (Oryz) PMM (XP_047395); Cryptosporidium parvum (Cpar) PMM (EAK87737); Encephalitozoon cuniculi (Ecu) PMM (CAD26542); Candida albicans (Calb) PMM (EAL02637); Pseudomonas aeruginosa (Pae) PMM/PGM (NP_254009); Streptococcus thermophilus (Sthe) PMM/PGM (AAD65380); Gluconacetobacter xylinus (Gxy) PGM (P38569); Corynebacterium glutamicum (Cglu) PGM (CAF21203); Vibrio fischeri (Vfis) PGM (AAM77720); Neisseria meningitides (Nmen) \(\beta\)-PGM (CAB85309); Enterococcus faecalis (Efae) \(\beta\)-PGM (NP_814693); and Lactococcus plantarum (Lplan) \(\beta\)-PGM (NP_783891). \(\alpha\)-PGM and HAD superfamilies are highlighted by light gray and dark gray areas, respectively. The white area indicates the cluster in the HAD superfamily that includes \(L.\) lactis \(\alpha\)-PGM.
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DISCUSSION

In this study, we have described the identification and functional analysis of L. lactis α-PGM, the first characterized member of a novel α-PGM family.

Sequence Comparison and Properties—All α-PGMs characterized so far belong to the α-D-phosphohexomutase superfamily of proteins (16). Interestingly, the α-PGM encoded by L. lactis pgmH has no sequence homology to members of the α-D-phosphohexomutase superfamily and does not contain the family’s consensus motifs. Instead, it shows sequence homology to eukaryotic PMMs (Figs. 1 and 3), an unrelated group of proteins that, despite their phosphohexomutase activity, belong to the HAD superfamily. The pgmH product features the conserved sequence motifs characteristic of the HAD superfamily (38, 39, 42, 43) and is similar in size to the eukaryotic PMMs (Fig. 1). The HAD superfamily comprises two branches that have acquired phosphohexomutase function, the eukaryotic α-PMMs (PF03332) and β-PGMs (PF00702) (11). As all known α-PGMs fall into the α-D-phosphohexomutase superfamily, we propose that the L. lactis α-PGM represents a novel line of α-D-phosphohexomutase evolution (Fig. 3).

Unlike the eukaryotic PMMs, which in general use both Man-1-P and Glc-1-P, L. lactis α-PGM shows strict specificity for α-Glc-1-P. Substrate specificity has been related to subtle residue variance in the catalytic domains (16). In the HAD superfamily, the catalytic cycle proceeds via a bisphosphorylated sugar intermediate to the reversible conversion of 1- to 6-phosphosugars and requires Mg2+ as a cofactor (Table 2) (43–45). Within this superfamily, the hexose C-1 configuration (α- or β-anomer) specificity and sequence/structural features allow us to distinguish between the eukaryotic α-PMMs and β-PGMs (Fig. 1) (12, 43, 46, 47). The position and fold of the cap domain (Fig. 1) place L. lactis α-PGM in HAD superfamily subclass II, whereas β-PGM is a subclass I protein. The sequence identities between L. lactis α-PGM (query sequence) and human α-PMM1 and L. lactis β-PGM are 25 and 10%, respectively. The structural and sequence similarities and the anomeric specificity suggest that L. lactis α-PGM is mechanistically closer to α-PMMs than to β-PGMs. This hypothesis is strengthened by the presence of the residues involved in the catalytic process of α-PMM1, in particular the nucleophile Asp9, the acid/base Asp10, and Gln51 (Asp19, Asp21, and Gln62 in human α-PMM1). However, only some of the conserved positively charged residues at the interface of the cap and core domains in α-PMMs are present in L. lactis α-PGM (Fig. 1), suggesting a mechanism of action different from that of the electrostatic wedge proposed for α-PMM (47).

A phylogram including both characterized and putative phosphohexomutases from eukaryotic and bacterial sources is given in Fig. 3 (48). The depicted topology clearly separates members of the α-D-phosphohexomutase and HAD superfamilies despite their similar function. The phylogenetic tree reflects the family division in the HAD superfamily, with bacterial β-PGMs and eukaryotic α-PMMs clustered into two distinct groups. L. lactis α-PGM is included in a cluster comprising proteins with unknown function of the human-associated organisms B. longum and P. acnes and the plant pathogen Gibberella zeae, which branches from the line leading to the eukaryotic α-PMMs. This suggests an origin common to these proteins and the eukaryotic α-PMMs, which also share the same α-anomeric specificity. A possible explanation relies on independent lateral gene transfer from eukaryotes to their commensal or pathogenic organisms. Surprisingly, of all the bacteria with available genome sequences, L. lactis appears to be unique insofar as it lacks an α-PGM of the α-D-phosphohexomutase superfamily.

Physiological Function of L. lactis α-PGM—In this work, we have shown that the highly specific α-PGM mediates the reversible conversion of α-Glc-1-P to Glc-6-P in L. lactis. Inactivation of α-PGMs is a trivial procedure in bacteria (6, 7, 49–55). Remarkably, all strategies attempted to disrupt pgmH failed. Our experiments with the pgmH conditional knock-out strain showed dramatic effects on growth rate and final biomass when the activity was below the control level. Although residual α-PGM activity could still sustain modest growth, cell division and morphology were affected as denoted by the appearance of long chains comprising cells that had lost their typical lactococcal shape (data not shown). L. lactis strains deficient in UDP-galactose 4-epimerase (gale) (56), the major autolysin (acmA) (57), or lipoteichoic acid α-acylation (58) shows a similar behavior, suggesting altered or deficient biosynthesis of cell wall polysaccharides. Earlier attempts to inactivate L. lactis MG1363 gallU, which encodes the enzyme that catalyzes the conversion of α-Glc-1-P to UDP-Glc, were also unsuccessful (56); and as for α-PGM, no other genes coding for a galactose uridylyltransferase were found in the available genome sequences (14). Therefore, it is reasonable to conclude that UDP-Glc synthesis in L. lactis relies entirely on pgmH and gallU gene products.

Overproduction of α-PGM affected the levels of glycolytic metabolites and the glycolytic flux from galactose, but not from glucose, reflecting the different role of pgmH in the metabolism of these two sugars: α-PGM is required for galactose degradation and was identified as a bottleneck in galactose metabolism, whereas its main function is providing precursors for biosynthetic pathways during growth on glucose. Altogether, the results presented here support the conclusion that pgmH is the only gene in the L. lactis genome coding for α-PGM activity. Therefore, the purification of a 65-kDa protein with α-PGM activity reported previously (9) can only be explained on the basis of the different genetic backgrounds used.

This work revealed a novel α-D-PGM that falls into the HAD family, unlike all other known α-PGMs. The demonstration of the essentiality of pgmH is a final confirmation of the crucial role played by the enzyme in the physiology of L. lactis. To further understand the unique features unraveled by this study, the structural determination of this new α-D-PGM is in progress.

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