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Glucokinase contributes to glucose phosphorylation in D-lactic acid production by *Sporolactobacillus inulinus* Y2-8

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Abstract *Sporolactobacillus inulinus*, a homofermentative lactic acid bacterium, is a species capable of efficient industrial D-lactic acid production from glucose. Glucose phosphorylation is the key step of glucose metabolism, and fine-tuned expression of which can improve D-lactic acid production. During growth on high-concentration glucose, a fast induction of high glucokinase (GLK) activity was observed, and paralleled the patterns of glucose consumption and D-lactic acid accumulation, while phosphoenolpyruvate phosphotransferase system (PTS) activity was completely repressed. The transmembrane proton gradient of 1.3–1.5 units was expected to generate a large proton motive force to the uptake of glucose. This suggests that the GLK pathway is the major route for glucose utilization, with the uptake of glucose through PTS-independent transport systems and phosphorylation of glucose by GLK in *S. inulinus* D-lactic acid production. The gene encoding GLK was cloned from *S. inulinus* and expressed in *Escherichia coli*. The amino acid sequence revealed significant similarity to GLK sequences from Bacillaceae. The recombinant GLK was purified and shown to be a homodimer with a subunit molecular mass of 34.5 kDa. Strikingly, it demonstrated an unusual broad substrate specificity, catalyzing phosphorylation of 2-deoxyglucose, mannitol, maltose, galactose and glucosamine, in addition to glucose. This report documented the key step concerning glucose phosphorylation of *S. inulinus*, which will help to understand the regulation of glucose metabolism and D-lactic acid production.

Keywords Glucokinase · *Sporolactobacillus inulinus* · Glucose metabolism · D-lactic acid production · Broad substrate specificity

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Introduction

Polylactic acid (PLA), a biodegradable polymer, is increasingly used as a substitute for conventional petroleum-based plastics. A stereocomplex polymer blend of poly(L-lactic acid) (PLLA) and poly(D-lactic acid) (PDLA) has better physical and thermochemical properties to overcome the brittle behavior of PLLA [32]. The blend of PLLA and PDLA also exhibits the greatest resistance to hydrolysis due to its strongest hydrogen-bonding and dipole–dipole interactions than pure PLLA and PDLA [12]. The emerging markets for PLA are likely to stimulate a significantly increased demand for high optical purity D-lactic acid. *Sporolactobacillus inulinus*, a homofermentative lactic acid bacteria, is widely used for industrial production of D-lactic acid of high optical purity, which efficiently ferments glucose exclusively to D-lactic acid [35, 36]. In our previous study, the highly efficient mutant strain *S. inulinus* Y2-8 produced 120–143 g/l D-lactic acid from 150 g/l glucose [33].

Phosphorylation of glucose is the first step of glycolysis for D-lactic acid production [27]. In eukaryotes and Archaea, cytoplasmic glucokinase (GLK) phosphorylates glucose irreversibly, triggers glucose utilization and plays an essential role in regulation of glucose metabolism

[6, 11]. In bacteria such as *Bacillus subtilis*, mainly two discrete pathways are employed for glucose uptake and phosphorylation. The first is phosphoenolpyruvate (PEP) phosphotransferase system (PTS). This is a transport system that catalyzes the coordinated uptake and conversion of glucose into glucose-6-phosphate (G6P) during transport [27]. The second is the GLK pathway in which glucose is transported by PTS-independent glucose transport systems such as a hexose:H^+ symporter that utilize chemiosmotic energy in the form of proton gradient. Glucose is then phosphorylated by cytoplasmic GLK [21, 27]. This second pathway probably acts under acidic growth conditions, in media containing high-concentration glucose. Continuous culture studies with *Streptococcus mutans* at a low pH and high concentration glucose showed that GLK activity was significantly increased under these conditions, while biosynthesis of the components of PTS was greatly repressed [4, 31]. Proteomic analysis of *S. mutans* confirmed up-regulated expression of GLK at a low pH [15]. Although phosphorylation of glucose is the key step in *S. inulinus* D-lactic acid production, little attention has been devoted to metabolic behavior. The purpose of this investigation was to increase our understanding of glucose metabolism involved in the uptake and phosphorylation of glucose in *S. inulinus* D-lactic acid production. For that purpose, we also cloned and characterized GLK that showed novel substrate flexibility.

Materials and methods

Chemicals, bacterial strains, plasmids and culture conditions

All commercially available chemicals were of reagent grade and purchased from Sigma-Aldrich or Roche Diagnostics, unless indicated otherwise. Molecular biology reagents and supplies were obtained from Takara or Invitrogen. *Sporolactobacillus inulinus* Y2-8, originally named *S.* sp. Y2-8, is a D-lactic acid high producing strain and deposited at China Center For Type Culture Collection (CCTCC No. 208052) [33]. The strains were cultivated under anaerobic conditions at 37 °C on agar medium. The seed culture medium was as described previously [33]. The fermentation medium was composed of (g/l): glucose (150), yeast extract (5), $MgSO_4$ (0.5), wheat bran (15), $CaCO_3$ (90), and 15 ml of corn steep liquor [pH 7.0]. *Escherichia coli* DH5α and plasmid pMD18 (TaKaRa, Japan) were used for cloning the gene encoding GLK (*glk*). *E. coli* BL21 (DE3) and plasmid pET-28a (Novagen) were used to express the recombinant GLK with a C-terminal His tag.

Fermentation

Batch fermentations of D-lactic acid were carried out in a 5-l fermentor (Biotech-2002, Shanghai Baoxing Inc. China) containing 3 l of the medium. Inoculum was prepared by growing on seed culture medium in a 500-ml flask at 37 °C and 150 rpm for 14 h. The fermentation medium was inoculated with 10 % (v/v) of seed culture, and maintained in anaerobic condition by sparging nitrogen gas (0.1 v.v.m) with agitation of 200 rpm. Samples were taken every 12 h for analyses.

Analytical methods

Analyses of cell growth, extracellular pH (pH_{ex}), concentration and optical purity of lactic acid were conducted as described previously [33]. Optical densities at 660 nm (OD_{660}) were converted to dry cell weight (DCW) according to the relationship between OD_{660} and DCW. The concentration of glucose was assayed with a Biosensor analyzer (SBA-40C; Institute of Biology, Shandong Academy of Sciences, China). After removing the most of $CaCO_3$ [33], cells at different growth stages were collected by centrifugation (8,000 × *g*, 10 min, 4 °C) to investigate intracellular pH (pH_i), GLK and PTS activities. The assay for pH_i was modified as described [25] using 2′,7′-bis-(2-carboxyethyl)-5(-and-6) carboxyfluorescein, acetoxymethyl ester (Beyotime Institute of Biotechnology, China) as a pH-sensitive indicator. Fluorescence was measured at 490-nm excitation and 535-nm emission wavelength (fluorescence spectrofluorometer F-7000, HITACHI, Japan). ΔpH was defined as transmembrane proton gradient (pH_i–pH_{ex}) expressed as pH units.

GLK and PTS assays

GLK activity was determined in cell-free extracts. The cells were disrupted by ultrasonication and centrifugated to eliminate cell debris (12,000 × *g*, 15 min, 4 °C), and the cell-free supernatant extracts were obtained. GLK activity was determined by a G6P dehydrogenase (GPD) coupled assay with $NADP^+$ as a cofactor [26]. The reaction was carried out in a mixture (250 µl) containing 50 mM Tris–HCl buffer [pH 7.0], 20 mM glucose, 25 mM $MgCl_2$, 2 mM $NADP^+$, 3 mM ATP, 1 U GPD and 0.5–5 µg of cell-free extract, or 0.1–1 µg of purified protein at 30 °C. This assay was used as standard activity test unless otherwise stated. All of the enzymatic assays were carried out by measuring the changes in the NADPH/NADH concentration through monitoring its absorbance at 340 nm. The protein concentration was determined according to Bradford using bovine serum albumin as a standard [3]. PTS

activity was assayed in cell suspension treated with 3 % (v/v) of toluene-ethanol (1:9, v/v) as previously described at 30 °C [14]. The assay mixture (250 µl) contained 100 mM potassium phosphate buffer [pH 7.0], 5 mM $MgCl_2$, 10 mM glucose, 0.2 mM NADH, 5 mM PEP, 2 U lactate dehydrogenase (LDH) and cells equivalent to 10–30 µg dry weight.

Cloning of the *glk* gene from *S. inulinus*

The highly conserved region of *glk* (approximately 400 bp) was amplified using two degenerate primers (5′-GGNGTT GACCTNGGCGGNACRA-3′ and 5′-CGCCAACNCCNG TNCCAAGYGT-3′) designed based on the nucleotide sequence alignment of different bacterial *glk*. This region showed obvious similarity to the fragments of *glk* from *Bacillus* spp. in GenBank. To determine the sequences upstream and downstream of conserved region, an efficient PCR strategy called thermal asymmetric interlaced (TAIL) PCR was used. The basic procedure and thermal cycling conditions were described by Nakayama [19]. Each set of nested specific primers was designed to exactly match a region of known sequence. Arbitrary degenerate primers were as in Mishra [17]. Sequence homology searches were performed using the BLAST program in NCBI (http://www.ncbi.nlm.nih.gov/) [2]. Multiple sequence alignment was performed using CLUSTAL X (version 1.8) [29]. Phylogenetic analysis was carried out using MEGA 4.0 software [28].

Expression and purification of GLK

The *glk* gene was amplified from the genomic DNA of *S. inulinus* Y2-8 using primers as follows: forward (5′-GC GCCATGGGCATTAATATTGGTGTA-3′ with an *Nco*I site (underlined) and reverse (5′-CTAAAGCTTCTTTA CGGTTGCACG-3′) with a *Hin*dIII site (underlined). The *Nco*I-*Hin*dIII-digested PCR-amplified fragment was cloned into the corresponding sites of pET-28a to produce recombinant plasmid pET-*glk*, which was then transformed into *E. coli* BL21(DE3). Overnight cultures of recombinant *E. coli* (pET-*glk*) were transferred into fresh medium and grown to mid-log phase. Isopropyl-*β*-D-thiogalactopyranoside (1 mM) was added to induce expression of GLK with a C-terminal His-tag and the incubation continued for 4 h at 37 °C. The recombinant cells were collected and cell-free extracts were obtained as described above. Recombinant His-tagged GLK was purified to homogeneity by Ni^{2+} affinity chromatography according to the manufacturer's instructions (GE Healthcare). The eluted solution was dialyzed in buffer (50 mM Tris–HCl [pH 8.0], 10 mM $MgSO_4$) for 3 h at 4 °C.

Molecular mass determination of GLK

The subunit molecular mass of purified GLK was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The molecular mass of native protein was analyzed by gel filtration chromatography on a Superdex 75 pg gel filtration column (GE Healthcare) eluted with 50 mM phosphate buffer [pH 7.2] and 150 mM NaCl. The Low Molecular Weight Gel Filtration Calibration Kit (GE Healthcare) was used for standard molecular size markers.

Characteristics and kinetic parameters of GLK

The effect of temperature on GLK activity was determined from 25 to 50 °C. The effect of pH was studied using different buffers for pH range of 3.0–10.5. The divalent cation and phosphoryl donor requirements were examined using the standard test system by exchanging either Mg^{2+} (4 mM) for other divalent cations (Mn^{2+}, Ni^{2+}, Ba^{2+}, Fe^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+}) or ATP (3 mM) for alternative phosphoryl donors (ADP, AMP, PEP, PPi and pyrophosphate) at equivalent concentrations. To determine the substrate specificity for sugars, hexokinase activity was determined by replacing glucose (20 mM) with other substrates (20 mM) (2-deoxyglucose, galactose, fructose, and so on) via a coupled assay with pyruvate kinase (PYK) and LDH [9]. The reaction mixture (250 µl) contained 20 mM hexose, 25 mM $MgCl_2$, 8 mM KCl, 2 mM NADH, 5 mM PEP, 3 mM ATP, 5 U PYK, and 6 U LDH in 50 mM Tris–HCl buffer [pH 9.0]. The regulation of GLK activity by various metabolites (G6P, ADP, AMP, phosphate, and citrate) (0.1, 1, 10 mM) was tested at different concentrations of substrates, ATP (0.1, 0.5, 1 mM) and glucose (0.5, 1, 5, 10 mM).

Results

Dynamic analysis of GLK and PTS activities during D-lactic acid production

Highly efficient D-lactic acid production by *S. inulinus* Y2-8 was obtained with a yield of 120 ± 4.1 g/l of D-lactic acid for 120 h and an optical purity of 99.1 % from initial concentration of 135 ± 2.6 g/l glucose (Fig. 1). The mutant Y2-8 is tolerant to high concentration of glucose because it was previously selected on high-concentration glucose agar plates after undergoing nitrogen ion-beam implantation [33]. During the first 24 h, glucose was predominantly consumed (0.63 ± 0.028 g/l/h) for fast cell growth and D-lactic acid was accumulated slightly. However, the amount of D-lactic acid was enough to result in

rapid decline of pH_{ex} from 6.8 to 5.3. Subsequently, pH_{ex} was maintained at about 5.0 by the neutralization of $CaCO_3$ to excessive D-lactic acid produced by the cells. Under these conditions, we found that the intracellular pH was maintained at 6.3–6.5. Thus, a large ΔpH of 1.3–1.5 units was formed, and it means the intracellular proton concentration was almost 20–30 fold higher than the extracellular one. Meanwhile, GLK activity markedly increased and reached a peak value of 665 ± 33 nmol/min/mg DCW at 36 h, which was almost 20-fold higher than that at the initial time. Particularly, glucose was consumed at an extraordinary rate of about 2.50 ± 0.32 g/l/h at 36–48 h, while GLK activity was maintained at an average high-level of 650 ± 30 nmol/min/mg DCW. After 48 h fermentation, GLK activity gradually fell to 455 ± 35 nmol/min/mg DCW and to 298 ± 25 nmol/min/mg DCW at the end of fermentation (i.e., 120 h) with decreased rates of cell growth and glucose consumption. On the contrary, PEP-dependent glucose phosphorylation was depressed with no obvious increase during the whole fermentation. The PTS activity was always lower than 40 ± 2.6 nmol/min/mg DCW, and after 24 h the value was merely one-tenth to one-twentieth of GLK activity. These results showed that GLK activity in *S. inulinus* was closely correlated with glucose consumption, cell growth and D-lactic acid production. Consequently, the GLK pathway contributed to the majority of glucose uptake and phosphorylation during D-lactic acid production, not the PTS pathway.

Cloning and sequence analysis of *glk* from *S. inulinus*

The *glk* gene from *S. inulinus* Y2-8 was cloned using degenerate PCR and TAIL PCR. The ORF of the *glk* gene (975 bp) corresponded to a protein of 324 amino acids with a predicted molecular mass of 33,955 Da. The sequence was deposited in the GenBank database under accession number JN860435 in October 2011. A putative RBS (AGGCGGT) was detected 8 bp upstream of the start codon. The closest upstream sequence (TTGGCA-17 bp-GAAAAT) similar to the σ^A-dependent promoter was identified [10].

BLASTP searches revealed the amino acid sequence of the *S. inulinus* GLK that showed 100 % identity with the putative GLK sequence from the genome sequence of *S. inulinus* strain CASD released at February 2012 [34]. The next highest amino acid sequence similarity (61 %) was to a putative GLK from *Bacillus pseudofirmus* OF4 (accession no. YP_003427745) and high similarities (50–61 %) to characterized GLKs from the family Bacillaceae, such as *Bacillus megaterium* (57 %) (CAA03848) and *B. subtilis* (50 %) (NP_390365). The *S. inulinus* GLK

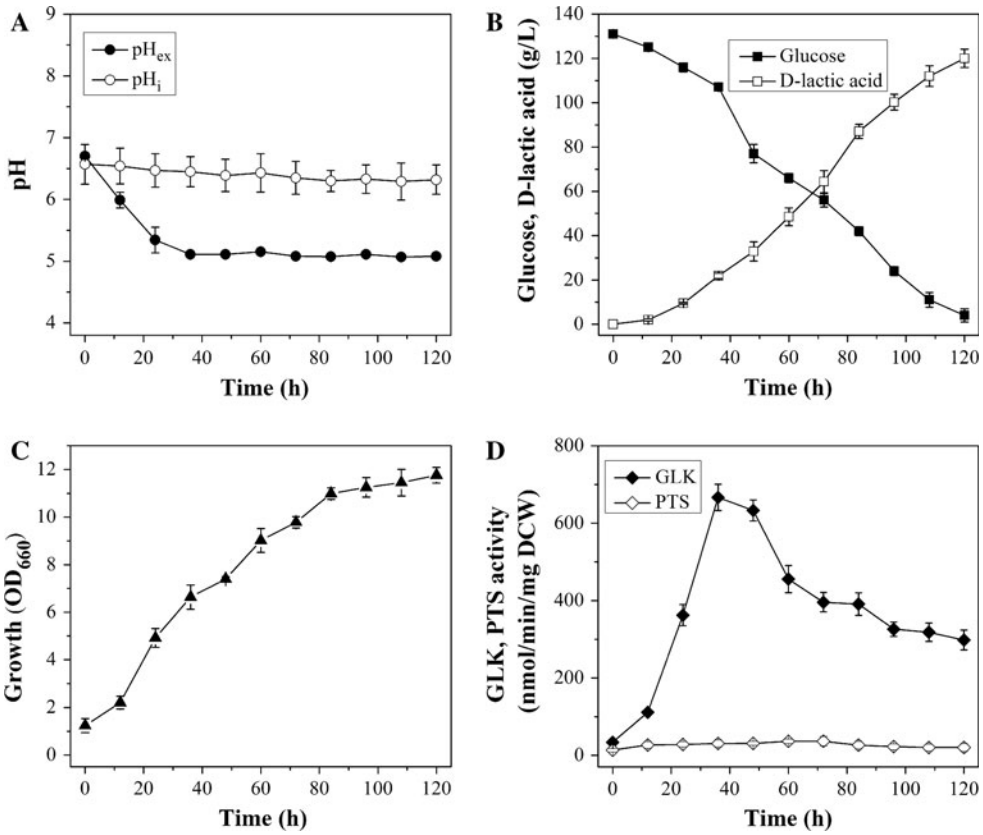

Fig. 1 Glucose phosphorylation, growth, and fermentation profiles of the *S. inulinus* D-lactic acid production. The fermentation of D-lactic acid by *S. inulinus* Y2-8 were carried out in a 5-l fermentor containing 3 l of the medium for 120 h. Samples were taken every 12 h for assays. The pH_{ex} and pH_i (**a**), concentration of residual glucose and D-lactic acid (**b**), cell growth (OD_{660}) (**c**), and GLK and PTS activities (**d**), were all measured as described in the Materials and methods section. Each value is the mean and standard deviation from duplicate runs of each batch

is a typical member of the ROK family (repressor protein, open reading frame, and kinases) for the presence of conserved motifs [5]. However, low similarities (20–34 %) were found with other members of the ROK family, including fructokinases (FRK/SCRK), N-acetylglucosamine kinases (NAGK), allose kinase (ALSK), beta-glucoside kinases (BGLK), xylose repressors (XYLR), and some ORFs with unknown function.

Expression and characteristics of GLK from *S. inulinus*

GLK from *S. inulinus* was heterologously expressed in *E. coli* and purified. The specific activity obtained from crude cell extract of recombinant *E. coli* (pET-*glk*) was approximately 30-fold higher than that of cells harboring pET-28a. The purified recombinant protein produced a single band at about 34.5 kDa following SDS-PAGE, in agreement with the predicted molecular mass (Fig. 2). The native GLK was determined to be 70 kDa by analytical gel filtration, indicating that GLK from *S. inulinus* forms a homodimer.

The optimum reaction temperature and pH of the *S. inulinus* GLK were 40 °C and 9.5–10.5, respectively. Under these conditions, the in vitro enzyme activity was about 45 % of its activity found at physiological pH (6.3–6.5) during fermentation. The K_m values for glucose and ATP were 4.26 and 1.03 mM, respectively, and the V_{max} value was 62.3 μmol/min/mg protein of the purified enzyme. GLK was inhibited by ADP with an inhibition constant *Ki* of 0.74 mM. None of the tested effectors (G6P, AMP, phosphate, and citrate) had a specific or regulatory effect on GLK activity. The enzyme required a divalent cation for activity and no GLK activity was detected in the absence of cations. Mg^{2+} was most effective at promoting catalysis and could partially be replaced by most of the divalent cations tested. The relative GLK activities in the presence of the cations were as follows: Mg^{2+}, 100 %; Mn^{2+} 52 %; Ni^{2+} 26 %; Ba^{2+} 22 %; Fe^{2+} 21 % and Ca^{2+} 20 %. When ATP was replaced by other phosphoryl group donors, such as ADP, AMP, PEP, PPi and pyrophosphate, or when no ATP was added, no GLK activity was observed. GLK could phosphorylate several sugars in addition to glucose to some extent. The relative activities were as follows: glucose 100 %; 2-deoxyglucose 73 %; mannitol 60 %; maltose 39 %; galactose 23 % and glucosamine 18 %. Other sugars tested, such as mannose, sucrose, sorbitol, myoinositol, cellobiose, arabinose and xylose, were not phosphorylated by purified GLK. The recombinant *S. inulinus* GLK with N-terminal His-tag or removed His-tag using thrombin showed the same property (data not shown). We conclude that the strictly ATP-dependent GLK from *S. inulinus* has broad cation and substrate specificities.

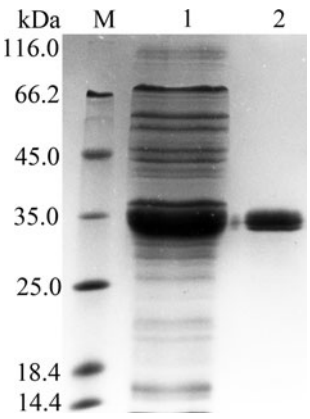

Fig. 2 SDS-PAGE of purified GLK from recombinant *E. coli* (pET-*glk*). *Lane 1*, crude cell extract from *E. coli* (pET-*glk*); *lane 2*, purified GLK; lane M, protein marker (Fermentas)

Discussion

Phosphorylation of glucose, the first key step of glycolysis, can be carried out by different pathways, dependent on organism and environment. GLK has been found in most organisms, while PTS is restricted to a minority of bacteria, and completely lacking in Archaea and eukaryotes [22]. It was reported that the growth environment of cells affects pathway used for the uptake and phosphorylation of glucose. Expression of GLK was up-regulated and expression of PTS was down-regulated in an acidic environment with a high concentration of glucose in *S. mutans* [4, 15, 31]. Our analyses during growth on high concentration glucose revealed that the GLK activity paralleled the patterns of glucose consumption, cell growth and D-lactic acid production in whole fermentation, while the PTS activity was repressed. The hexose:H^+ symporter is presumably energized to internalize glucose continuously by huge proton motive force [21]. The huge transmembrane proton gradient in *S. inulinus* D-lactic acid production was expected to generate a large proton motive force to the uptake of glucose though PTS-independent transport systems. At this time, non-PTS cytoplasmic glucose was rapidly phosphorylated by induced GLK. Subsequently, glucose was mainly phosphorylated by GLK directly for high efficient D-lactic acid production. Thus, it became clear that the GLK pathway was the major route of glucose utilization and GLK plays a vitally important role in the first irreversible step of glycolysis leading to D-lactic acid production.

GLK from *S. inulinus* demonstrated broad cation specificity for most divalent cations tested could be used to various extents. Generally, Mg^{2+}, Mn^{2+} and Co^{2+} are the most efficient cations for catalysis, such as GLKs from

Bacillus stearothermophilus [7] and *Aeropyrum pernix* [9]. Curiously, the *S. inulinus* GLK revealed no activity with Co^{2+}.

GLK from *S. inulinus* showed unique broad substrate specificity compared to all other characterized bacterial GLKs. A comparison of the substrate specificity of characterized GLKs from bacteria, Archaea and eukaryotes is given in Table 1. GLK from different branches of life had different characteristic in molecular mass, phosphoryl donor specificity and substrate specificity. The bacterial GLKs show a high specificity [16, 26] except GLK from *Bacillus sphaericus*, which is considered to be an archaic bacterium [8]. Nevertheless, GLKs from eukaryotes and Archaea usually exhibit broad specificities for sugars [9, 30]. Interestingly, the substrate specificity of the *S. inulinus* GLK was similar to that of GLKs from eukaryotes and Archaea, exhibiting broad substrate specificity for sugars. Surprisingly, GLK from *S. inulinus* could also catalyze phosphorylation of maltose. It is known that hexokinases (HK) and GLKs can phosphorylate a range of structurally distinct glucose epimers, but none has yet reported as able to phosphorylate a disaccharide. The broad substrate specificity of the enzyme renders the potential utilization of abundant complex carboxydrates in plant biomass by *S. inulinus*.

The finding of unique substrate flexibility of the *S. inulinus* GLK led us to conduct phylogenetic study using the Minimum Evolution method (Fig. 3). The 35 selected protein sequences comprised enzymes with representative functions in the ROK family. The tree consisted of 11 well-supported large clades and some individual clades, being consistent with their functions and taxa and representing a highly divergent set of enzymes. The tree demonstrated that GLK from *S. inulinus* has a very close evolutionary relationship with GLKs from Firmicutes. However, all characterized GLKs in this subgroup revealed rigorous specificity for glucose, except the *S. inulinus* GLK [23, 26]. It is widely believed that primordial enzymes probably had broad substrate specificity, allowing catalysis of a wide range of substrates [1]. Kawai et al. [13] proposed that glucomannokinase from *Arthrobacter* sp. could be the common ancestor based on its simple structure and ancient phosphoryl donor, poly(P). Our results revealed that the GLK from *S. inulinus* was a more likely candidate for an ancestor of the ROK family, based on the broad substrate specificity, allowing adaptation to different environments, and located near the base of the tree with a short branch length. However, the molecular mechanism of the broad substrate specificity for GLK from *S. inulinus* is still puzzling, and further studies on the crystal structure of the protein might provide an explanation.

Table 1 Comparison of substrate specificity of GLKs and HKs

Taxon	Species	Enzyme	Nucleotide specificity	Mr (kDa)	Relative activity								Reference
					Glc	2DG	Gal	Fru	Man	Mtl	GlcN	Malt	
Bacteria	*Sporolactobacillus inulinus*	GLK	ATP	34	100	73	23	6	ND	60	18	39	This work
	Bacillus subtilis	GLK	ATP	34	100	–	ND	ND	ND	–	–	–	[26]
	Bacillus stearothermophilus	GLK	ATP	34	100	ND	ND	ND	ND	–	ND	ND	[7]
	Streptococcus mutans	GLK	ATP	34	100	ND	ND	ND	ND	ND	ND	ND	[23]
	Bacillus sphaericus	GLK	ATP	33	100	–	–	40	48	–	–	–	[8]
	Arthrobacter sp.[a]	GMK	Poly (P)/ATP	30	100	–	ND	ND	66/33	–	ND	–	[18]
	Escherichia coli	GLK	ATP	35	100	–	ND	ND	ND	–	–	–	[16]
Archaea	*Aeropyrum pernix*	GLK	ATP	36	100	129	–	43	54	–	77	–	[9]
	Thermoproteus tenax	HK	ATP	33	100	173	ND	214	39	–	–	–	[6]
	Methanococcus jannaschii	GLK/PFK	ADP	53	100	19	ND	14	ND	–	ND	–	[24]
Eukarya	*Aspergillus niger*	GLK	ATP	55	100	73	ND	1	55	ND	26	1	[20]
	Rat	HK I	ATP	98	100	–	ND	110	ND	–	–	–	[30]
		HK II	ATP	96	100	–	140	120	120	–	–	–	
		HK III	ATP	100	100	–	100	130	110				
		GK	ATP	49	100	–	40	20	80				

Mr Molecular mass, *Glc* glucose, *2DG* 2-deoxyglucose, *Gal* galactose, *Fru* fructose, *Man* mannose, *Mtl* mannitol, *GlcN* glucosamine, *Malt* maltose, *ND* not detected, – not tested

[a] Activity measured using poly(P) or ATP, respectively

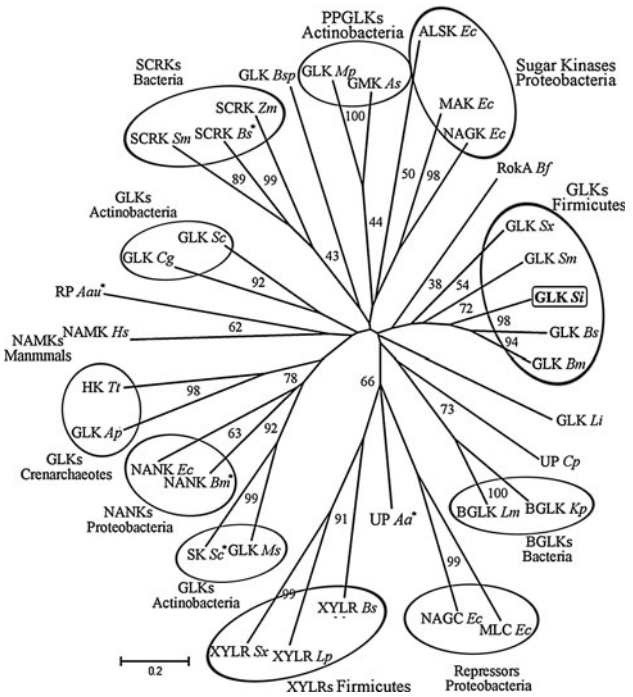

Fig. 3 Phylogenetic tree of the ROK family. Bootstrap proportions are shown at branching points according to the minimum evolution method; only values greater than 30 % are shown. Sequence accession numbers are shown in *parentheses*. GLK *Si*, GLK from *S. inulinus* (JN860435), is in *boldface* and enclosed in *box*. The putative sequences are marked with an *asterisk*. GLK: *Sx*, *Staphylococcus xylosus* (CAA59069); *Sm*, *S. mutans* (AAN58285); *Bs*, *B. subtilis* (NP_390365); *Bm*, *B. megaterium* (CAA03848); *Li*, *Leptospira interrogans* (AAN48636); *Ms, Mycobacterium smegmatis* (YP_885749); *Ap*, *A. pernix* (NP_148378); *Cg*, *Corynebacterium glutamicum* (AAF80161); *Sc*, *Streptomyces coelicolor* (CAA46727); *Bsp*, *B sphaericus* (ABK63288); *Mp*, *Microlunatus phosphovorus* (BAC84981); UP, unknown protein; *Cp*, *Clostridium perfringens* (P26832); *Aa*, *Acetohalobium arabaticum* (YP_003826920); BGLK: *Kp*, *Klebsiella pneumoniae* (Q93LQ8); *Lm*, *Listeria monocytogenes* (Q8Y3R9); MLC *Ec*, making large colonies protein from *E. coli* (P50456); NAGK *Ec*, (P7595); XYLR, *Bs* (AAA 22896); *Lp*, *Lactobacillus pentosus* (AAA25257); *Sx* (CAA40823); SK, sugar kinase; *Sc* (NP_625371); NANK, N-acetylmannosamine kinase, *Ec* (P45425); NANK *Bm* and NANK *Hs*, NANK functional domain from *Brucella melitensis* (Q8YBP2) and *Homo sapiens* (Human) (Q9Y223); HK, *Tt*, *Thermoproteus tenax* (CAD52839); RP, repressor protein, *Aau*, *Arthrobacter aurescens* (YP_948090); SCRK, *Sm* (BAA02467); *Bs* (O05510); *Zm*, *Zymomonas mobilis* (AAA27687); GMK *As*, glucomannokinase from *Arthrobacter* sp. (BAC78847); ALSK *Ec*, (P32718); MAK *Ec*, Manno(fructo) kinases from *E. coli* (P23917); NAGC *Ec*, N-acetylglucosamine repressors from *E. coli* (P0AF20); RokA *Bf*, ROK protein from *Bacteroides fragilis* (AAV80416)

In summary, on the physiological level it was found that the GLK pathway was the major route of glucose uptake and phosphorylation in *S. inulinus* D-lactate production. GLK was prominently up-regulated followed by the formation of a large transmembrane proton gradient, while the PTS pathway was completely repressed. Furthermore, the key enzyme GLK was characterized, which differed from other bacterial GLKs in its broad substrate specificity. This study advances our understanding of regulation in D-lactic acid production by the efficiently industrial bacterium *S. inulinus*.

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