Structure-Based Enhancement of the First Anomeric Glucokinase

Jie Yang, Lesley Liu, and Jon S. Thorson*[a]

Many pharmaceutically important compounds derive from carbohydrate-containing natural products, and sugar moieties of these molecules have been proven to play an important role in drug targeting, biological activity, and pharmacology.[1–7] Thus, altering the glycosylation of natural products would significantly contribute to the diversity of novel therapeutics. Among a number of routes for altering glycosylation, natural-product glycorandomization is one of the most efficient approaches for complex secondary metabolites.[8–14] In vitro glycorandomization (IVG) technology establishes a promiscuous chemoenzymatic system to quickly build diverse glycorandomized libraries based on natural-product structures (Figure 1 a). This process utilizes chemical synthesis to provide a repertoire of unique sugar precursors, and three promiscuous enzymes to activate (anomeric sugar kinases and nucleotidylyltransferases) and attach (glycosyltransferases) these carbohydrate libraries to various complex natural-product aglycons. Anomeric sugar kinases, as key components of glycorandomization, directly determine the availability of sugar phosphates for chemoenzymatic routes toward complex glycoconjugates. Thus, generation of a flexible sugar kinase capable of accepting a wide array of monosaccharide substrates would directly enhance the efficiency of IVG. Of particular interest are D-*gluco*-configured scaffolds given their prevalence in glycosylated natural products (Figure 1 b),[1,3,4,6] glycoproteins,[15–18] as well as many bacterial and eukaryotic cell-surface glycosylation patterns.[18–20]

To date, an enzyme capable of D-glucose (Figure 2, **13**) anomeric phosphorylation (a glucokinase, or GlcK) remains elusive. Toward this goal, directed evolution of *E. coli* galactokinase (GalK) led to the Y371H variant with remarkably widened substrate flexibility at C-2, C-3, and C-5 of the sugar. Yet, all sugars tested containing alternative C-4 substitutions (e.g. 4-deoxy-D-galactose, **12** or **13**, Figure 2) were not accepted as substrates for the evolved catalyst.[21] As an alternative approach, the recently elucidated structure of *Lactococcus lactis* GalK provides a template for rational sugar kinase engineering.[22] The *L. lactis* GalK structure reveals that the strongly conserved active-site residues Asp45 and Tyr233 hydrogen bond with the critical galactose C-4 axial hydroxyl.[22] While a recent saturation mutagenesis of the equivalent residues within the *E. coli* GalK revealed the role of the active-site tyrosine to be hydrophobic stacking and expanded the substrate range to include **12**,[23] a GlcK has still not been found.

To continue our quest for an anomeric GlcK and further explore the relevance of the *L. lactis* structural model to other GalKs, we report the generation and characterization of the Y385H *L. lactis* GalK mutant—the promiscuous *E. coli* equivalent of which was discovered through directed evolution.[21] Remarkably, we reveal that this *L. lactis* variant displays a substantial degree of kinase activity toward several C-4-substituted sugars, including **12**, **13** and 4-azido-4-deoxy-D-galactose (Figure 2, **14**). Moreover, complete characterization of the *L. lactis* wild-type GalK surprisingly revealed inherent GlcK activity. As such, this is the first reported naturally occurring GlcK, the efficiency of which was improved by engineering with our previous *E. coli* GalK-directed evolution studies as a template. In addition, these studies suggest that enzyme species/source differentiation may present general advantages in optimizing enzymes for synthetic purposes.

The *L. lactis* Y385H mutant (Y385H) was generated through PCR primer-directed site-directed mutagenesis. By using this approach, plasmid pET28b-*galkY385H* was constructed, and soluble over-expression of *L. lactis* GalK Y385H was accomplished in *E. coli* BL21 (DE3). To compare the substrate specificities of wild-type and mutant enzymes, freshly purified wild-type and mutant (Y385H) enzymes were screened against a sugar library consisting of 30 putative monosaccharide substrates. Typically for each sugar, enzymatic reactions containing the wild-type or Y385H mutant, were monitored by the DNS assay in triplicate.[24] Controls in the absence of enzyme or sugar were also performed in parallel and all sugar-1-P products were confirmed via MS/MS analysis.

Figure 2 illustrates the substrate profiles of wild-type *L. lactis* GalK and the *L. lactis* Y385H GalK mutant. The *L. lactis* wild-type enzyme accepted 13 sugar substrates, a striking expansion of the typically limited substrate scopes observed for the GalK family.[24–27] Among these new monosaccharide substrates were three notable C-4-substituted sugars (**12**, **13**, and **14**) and two unique L-configured sugars (L-altrose, **22**, and L-glucose, **23**), all of which failed as active substrates for native GalKs from other sources.[24–27] As expected, the *L. lactis* Y385H mutant was even more promiscuous with several new structures (Figure 2, 2-deoxy-D-glucose, **15**, 6-amino-6-deoxy-D-galactose, **17**, and 6-azido-6-deoxy-D-galactose, **19**) also weakly recognized by this variant. Yet, while the *L. lactis* Y385H mutant displayed slightly enhanced activities with respect to most observed *L. lactis* wild-type GalK substrates (Table 1 and

[a] *J. Yang, Dr. L. Liu, Prof. J. S. Thorson*
University of Wisconsin–Madison, School of Pharmacy
Laboratory for Biosynthetic Chemistry
777 Highland Avenue, Madison, Wisconsin 53705 (USA)
Fax: (+1) 608-262-5345
E-mail: jsthorson@pharmacy.wisc.edu

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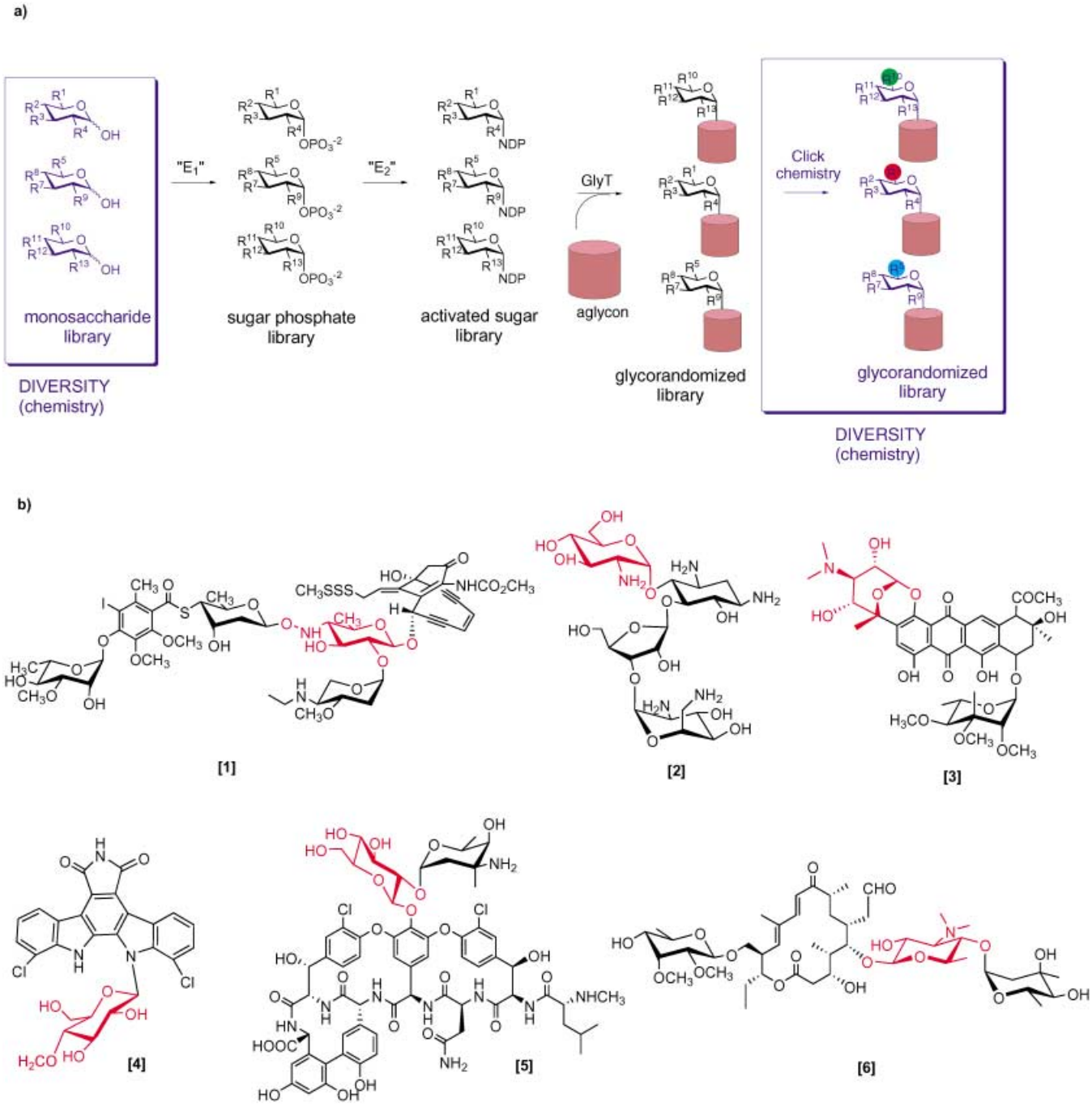

Figure 1. *a) Schematic for natural product in vitro glycorandomization illustrating the great potential for enhanced diversity through the simplistic bypass of the many specific SLBP enzymes. In IVG, the diversity stems from the upstream chemical synthesis of novel monosaccharides and downstream chemical modification, via chemical ligation strategies. The core of IVG is dependent upon three key enzymes (E_1, kinase; E_2, nucleotidylyltransferase; and GlyT, glycosyltransferase) to accomplish the difficult sugar-activating and -attachment chemistry in a stereo- and regiospecific manner. b) Representative examples of D-glucose-configured sugar (highlighted in red) containing natural products used as therapeutics or agricultural agents: calicheamicin (**1**), paromomycin (**2**), nogalomycin (**3**), rebeccamycin (**4**), vancomycin (**5**), and tylosin (**6**).*

Figure 2), the extent of enhancement was not nearly to the level invoked upon the *E. coli* GalK by the equivalent mutation (Y371H).[21] In the context of synthetic application, it is also interesting to note that while the *L. lactis* GalK Y385H activities in the presence of many sugars vary drastically (Table 1), the final sugar-1-P yields in representative chemoenzymatic reactions are often >40% (e.g. **7**–**11**, **13**, **22**, and **23**, Figure 2).

To better understand the nature of this mutation in the context of the *L. lactis* enzyme, the kinetic parameters for the representative substrates **7** and **13** with the wild-type *L. lactis* GalK and the *L. lactis* Y385H mutant were determined (Table 2).

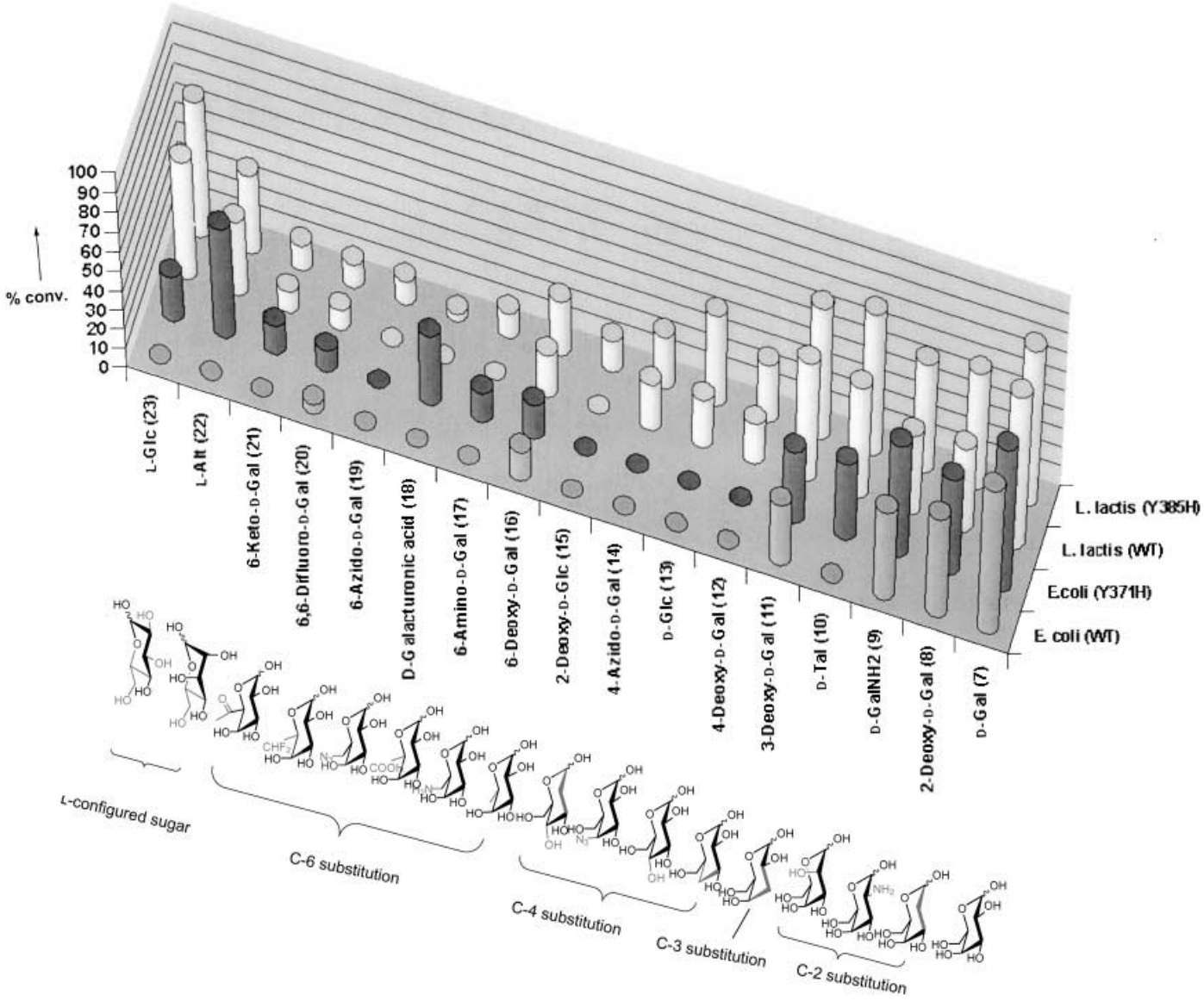

Figure 2. *Conversion (%) of sugar substrates by wild-type and mutant GalKs. For each enzymatic reaction: [sugar] = 8 mM, [ATP] = 15 mM, [$MgCl_2$] = 5 mM, [enzyme] = 5.0 μM and reaction time = 180 min. Percent conversion was calculated as described in the Experimental Section.*

Consistent with previous studies on the *E. coli* Y371H "equivalent" mutant,[21] these kinetic studies revealed that the *L. lactis* Y385H mutant enzyme also displays remarkably enhanced k_{cat} values for the native substrate **7** with a slightly decreased substrate affinity. The case for **13**, however, contrasts this general trend (also noted with the *E. coli* variant).[21] For **13**, the *L. lactis* Y385H mutant and wild-type GalKs display similar k_{cat} values but the affinity for **13** is enhanced approximately twofold (Table 2).

In conclusion, equivalent point mutations (Tyr→His) in both *E. coli* and *L. lactis* GalKs invoke enhanced catalytic efficiency (k_{cat}/K_m) and substrate promiscuity. Notably, this work reveals the first naturally occurring GlcK and the most promiscuous anomeric sugar kinase reported to date (*L. lactis* Y385H with >17 different sugar substrates) and suggests the future combined application of directed evolution, rational engineering, and new species/sources hold significant promise in optimizing these catalysts further for synthetic purposes.

Experimental Section

General: ^1H and ^{13}C NMR spectra were recorded in D_2O on a Varian 400 MHz spectrometer. Optical rotation was measured by using a Perkin Elmer 241 polarimeter.

Materials: *E. coli* strains XL1-blue and BL21 (DE3) were purchased from Stratagene (La Jolla, CA). All reagent grade chemicals and enzymes were purchased from Promega (Madison, WI), Sigma (St. Louis, MO), Fisher/Acros Organics (Hanover Park, IL), or Fluka (Milwaukee, WI).

Chemical synthesis of unnatural sugar compounds: 6-deoxy-6,6-difluoro-D-galactose **20**,[28] 6,7-dideoxy-D-*galacto*-hept-6-ynopyra-

Table 1. Representative activities and product mass. For each enzymatic reaction: [sugar] = 8 mM, [ATP] = 15 mM, [$MgCl_2$] = 5 mM. (–, no conversion; ~, not determined.)

Sugar substrate	Wild type Initial velocity [μmol min^{-1} mg^{-1}]	Y385H Initial velocity [μmol min^{-1} mg^{-1}]	Product MS Calculated	Determined $[M-H]^-$
D-Gal (**7**)	1.44 (±0.12)	2.14 (±0.09)	260.0	258.9
2-deoxy-D-Gal (**8**)	0.68 (±0.09)	0.76 (±0.05)	244.0	242.9
D-GalNH2 (**9**)	0.31 (±0.07)	0.48 (±0.08)	259.1	257.9
D-Tal (**10**)	0.20 (±0.04)	0.36 (±0.04)	260.0	258.9
3-deoxy-D-Gal (**11**)	0.26 (±0.05)	0.30 (±0.04)	244.0	242.9
4-deoxy-D-Gal (**12**)	0.06 (±0.02)	0.09 (±0.01)	244.0	242.9
D-Glc (**13**)	0.08 (±0.02)	0.12 (±0.04)	260.0	258.9
4-azido-D-Gal (**14**)	0.07 (±0.01)	0.07 (±0.02)	285.0	283.9
2-deoxy-D-Glc (**15**)	–	0.06 (±0.02)	244.0	243.0
6-deoxy-D-Gal (**16**)	0.15 (±0.03)	0.21 (±0.04)	244.0	242.9
6-amino-D-Gal (**17**)	–	0.08 (±0.02)	259.1	257.9
D-galacturonic acid (**18**)	–	0.02 (±0.01)	274.0	~
6-azido-D-Gal (**19**)	–	0.05 (±0.01)	285.0	283.9
6,6-difluoro-D-Gal (**20**)	0.04 (±0.01)	0.04 (±0.01)	280.0	~
6-keto-D-Gal (**21**)	0.04 (±0.01)	0.05 (±0.02)	272.0	~
L-Alt (**22**)	0.22 (±0.05)	0.24 (±0.06)	260.0	258.9
L-Glc (**23**)	0.23 (±0.03)	0.27 (±0.07)	260.0	258.9

Table 2. Kinetics of GalK variants with D-Gal and D-Glc.

	D-Gal (**7**)			D-Glc (**13**)		
	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [mM^{-1} min^{-1}]	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [mM^{-1} min^{-1}]
WT GalK (*L. lactis*)	6.4 (±0.6)	116 (±4.0)	18.1 (±2.4)	14.1 (±1.1)	10.2 (±1.2)	0.7 (±0.1)
Y385H (*L. lactis*)	9.0 (±0.7)	318 (±6.0)	35.3 (±3.3)	7.2 (±0.6)	10.3 (±1.0)	1.43 (±0.2)
WT GalK (*E. coli*)	2.1 (±0.4)	108 (±3.0)	51.4 (±9.8)	–[a]	–	–
Y371H (*E. coli*)	5.6 (±0.3)	220 (±9.0)	39.3 (±3.6)	–	–	–

[a] – = no conversion.

nose,[28] 6-bromo-6-deoxy-D-galactose,[29,30] and 6-chloro-6-deoxy-D-galactose[29,31] were prepared as previously described.

7-deoxy-D-*galacto*-heptos-6-ulose **21**: 7-deoxy-1,2:3,4-di-*O*-isopropylidene-D-*galacto*-heptopyranose-6-ulose was prepared as previously described.[32] Methanolic sulfuric acid (1% *v/v*, 100 mL) and H_2O (200 mL) were added sequentially to a stirred solution of 7-deoxy-1,2:3,4-di-*O*-isopropylidene-D-*galacto*-heptopyranose-6-ulose[32] (2.98 g, 10.9 mmol) in MeOH (100 mL). The solution was stirred for 3 h at 100 °C, after which time it was cooled and then neutralized by the addition of aqueous KOH (10 M). The solution was evaporated to dryness under reduced pressure. The residue was taken up in MeOH and filtered. The filtrate was concentrated under reduced pressure, and the residue was subjected to flash chromatography by using CH_2Cl_2/MeOH (9:1) to give the free sugar **21** as a white solid (0.67 g, 32%, α/β 2:1), TLC $R_f = 0.60$ in isopropanol/ethyl acetate/water (7:1:2); $[\alpha]_D^{25} = +0.60$ ($c = 0.86$ in H_2O). For the α-anomer: ^{1}H NMR: $\delta = 5.30$ (d, $J_{1,2} = 4$, 1H; H-1), 4.73, (d, $J_{4,5} = 1.2$, 1H; H-5), 4.44 (dd, $J_{3,4} = 3.2$, 1H; H-4), 3.93 (dd, $J_{2,3} = 10.4$, 1H; H-3), 3.77 (dd, 1H; H-2), 2.24 ppm (s, 3H; H-7); ^{13}C NMR: $\delta = 209.7$, 92.6, 76.4, 70.0, 69.1, 68.1, 26.4 ppm. For the β-anomer: ^{1}H NMR: $\delta = 4.58$ (d, $J_{1,2} = 8$, 1H; H-1), 4.36 (d, $J_{4,5} = 1.2$, 1H; H-5), 4.34 (dd, $J_{3,4} = 3.6$, 1H; H-4), 3.70 (dd, $J_{2,3} = 9.6$, 1H; H-3), 3.47 (dd, 1H; H-2), 2.24 ppm (s, 3H; H-7); ^{13}C NMR: $\delta = 209.1$, 96.5, 80.2, 72.8, 71.6, 69.6, 26.5 ppm; MS(ES): calcd. for $C_7H_{12}O_6$ 192.2, found *m/z* 190.9 $[M-H]^-$.

Site-specific mutagenesis: The plasmid containing the desired point mutation was constructed by using *PfuUltra*™ high-fidelity DNA polymerase for mutagenic primer-directed replication with the wild-type plasmid pET28b-*galk* as template.[33,34] Pairs of designed primers both included the desired modification (primer sequences = 5′-CCAGCAAGCTTTCACGTCGCTCAAATTGGTTCTG-GT-3′/5′-ACCAGAACCAATTTGAGCGACGTGAAAGCTTGCT-GG-3′). Amplification was accomplished under the following conditions: pET28b-*galk* plasmid DNA (40 ng), oligonucleotide primer (each 120 ng), dNTPs mixture (1 μL, 2.5 mM), 2.5 units of *PfuUltra*™ high-fidelity DNA polymerase, in a total volume of 50 μL. Thermocycler parameters: initial denaturation, 2 min at 95 °C; amplification, 12 cycles, 0.5 min at 95 °C, 1 min at 55 °C, 6.5 min at 68 °C; terminal hold, 5 min at 68 °C. The amplified plasmids were treated with *Dpn*I to digest the parental DNA template, and the mutated prodigy plasmid subsequently used to transform *E. coli* XL1-blue. The desired point mutation was verified by sequencing.

Enzyme expression and purification: *E. coli* BL21 (DE3) cells were transformed with pET28b-*galk* or pET28b-*galkY385H* and grown on an LB_{Kan30} plate overnight at 37 °C. A single colony was transferred into LB_{Kan30} medium (4 mL) and grown at 37 °C overnight, an aliquot of which (1 mL) was used to inoculate 100 mL of LB_{Kan30} medium and incubated with shaking at 37 °C. At $OD_{600} = 0.8$, the cells were cooled on ice for 5 min and transferred to a 16 °C shaker. Then, IPTG (1 M) was added (1 mM final concentration), and the culture was incubated with shaking (140 rpm) for 20 h. The *L. lactis* wild-type GalK and variant Y385H were purified by using metal affinity chromatography on Ni-NTA Spin Columns (QIAGEN, Valencia, CA). The concentration of protein was determined by Bio-Rad protein assay.

Characterization of *L. lactis* wild-type GalK and Y385H: The DNS assay was used to determine the substrate specificity of wild-type GalK and variant (Y385H) as previously described.[24] Standard curves for each sugar and progress curves were established as described.[24] The percent conversion for each reaction was calculated by using Equation (1),

$$\% \text{ conversion} = [(C_0 - C_{180})\, C_0] \times 100 \quad (1)$$

where C_0 represents the sugar concentration in the reaction system at starting point, and C_{180} represents the sugar concentration in the reaction system after 180 min. The initial velocity for

each active substrate was determined by the slope value of the linear phase in the progress curve. At least two independent experiments were performed for each sugar substrate. After 180 min, the enzymatic reaction was quenched with MeOH and centrifuged (10 min, 12 000 rpm), then the supernatant (diluted 20-fold) was submitted for MS and MS/MS analysis. The kinetic data were determined and analyzed as described.[24]

Note added in proof: While this manuscript was under review, David W. Rice and co-workers reported the crystal structure of the *Pyrococcus furiosus* galactokinase and found Tyr200 (equivalent to Tyr223 in *E. coli* GalK) to hydrogen bond with the C-4-OH of D-galactose.[35]

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