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Discovery of a Kojibiose Phosphorylase in *Escherichia coli* K-12

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Abstract

The substrate profiles for three uncharacterized enzymes (YcjM, YcjT, and YcjU) that are expressed from a cluster of twelve genes (*ycjM-W* and *ompG*) of unknown function in *Escherichia coli* K-12 have been determined. Through a comprehensive bioinformatic and steady-state kinetic analysis, the catalytic function of YcjT was determined to be kojibiose phosphorylase. In the presence of saturating phosphate and kojibiose (α-(1,2)-D-glucose-D-glucose) this enzyme catalyzes the formation of D-glucose and β-D-glucose-1-phosphate (k_{cat} = 1.1 s^{-1}, K_m = 1.05 mM, and k_{cat}/K_m = 1.12 x 10^3 M^{-1} s^{-1}). Additionally, it was also shown that in the presence of β-D-glucose-1-phosphate, YcjT can catalyze the formation of other disaccharides using 1,5-anhydro-D-glucitol, L-sorbose, D-sorbitol or L-iditol as a substitute for D-glucose. Kojibiose is a component of cell wall lipoteichoic acids in Gram-positive bacteria and is of interest as a potential low-calorie sweetener and prebiotic. YcjU was determined to be a β-phosphoglucomutase that catalyzes the isomerization of β-D-glucose-1-phosphate (k_{cat} = 21 s^{-1}, K_m = 18 μM, and k_{cat}/K_m = 1.1 x 10^6 M^{-1} s^{-1}) to D-glucose-6-phosphate. YcjU was also shown to exhibit catalytic activity with β-D-allose-1-phosphate, β-D-mannose-1-phosphate, and β-D-galactose-1-phosphate. YcjM catalyzes the phosphorolysis of α-(1,2)-D-glucose-D-glycerate with a k_{cat} = 2.1 s^{-1}, K_m = 69 μM, and k_{cat}/K_m = 3.1 x 10^4 M^{-1} s^{-1}.

Graphical abstract

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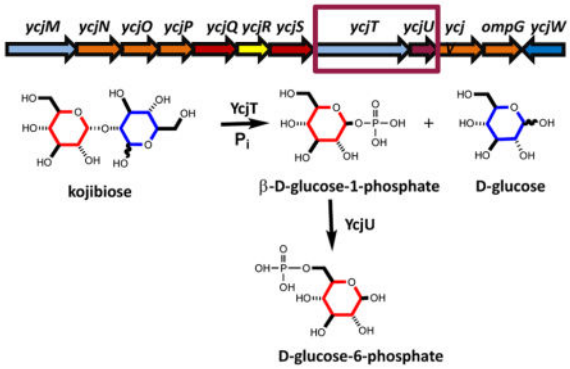
Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the AS Publications website at DOI:

This information includes: Scheme S1: Structures of disaccharides tested as substrates for YcjM and YcjT; Scheme S2: Structures of monosaccharides tested as substrates for YcjM and YcjT; Scheme S3: Structures of sugars tested as substrates for YcjM and YcjT; Scheme S4: Structures of deoxy-D-glucose variants and inositols tested as substrates for YcjM and YcjT; Scheme S5: Reactions catalyzed by YcjU; Figure S1: Homology model for active site of YcjM built using Phyre2. Figure S2: Multiple sequence alignment of YcjT, and kojibiose phosphorylases from various sources.



INTRODUCTION

In a typical adult there are more microbial prokaryotic cells (~3.8 x 10^{13}) than there are human eukaryotic cells (~3.0 x 10^{13}).[1] These bacteria reside predominantly in the gastrointestinal tract and are strongly influenced by factors such as diet, disease, and antibiotic usage.[2–4] The impact of these bacterial communities on human health has been investigated and a wide range of diseases such as obesity, diabetes, autoimmune disorders, inflammatory bowel disease, colon cancer, and transmission of retroviruses has been linked to dysbiotic microbiota.[5–14] Since the first bacterial genome was sequenced, there have been rapid technological advancements in the rate of bacterial genome sequencing.[15, 16] Metagenomic sequencing projects have led to the creation of a 'gene catalogue' of the most common microbial genes.[17] The 'microbiome' encode for a significant number of enzymes that are lacking in the host glycobiome. These microbial enzymes often confer metabolic capabilities to the host, such as the breakdown of indigestible polysaccharides.[18] However, the functional annotation of the microbial enzymes found in these organisms has not kept pace with the speed of various DNA sequencing projects.

A cluster of 12 genes (*ycjM-W* and *ompG*) has been identified in *Escherichia coli* K-12 MG1655, which may code for the expression of enzymes used for the metabolism of carbohydrates of unknown structure (Figure 1). Most of the enzymes in this putative biochemical pathway bear little similarity to proteins of known metabolic functions. This gene cluster is also conserved in a number of other gut-dwelling, Gram-negative bacterial species, including *Salmonella enterica*, *Shigella dysenteriae*, *Enterobacter tasmaniensis*, and *Citrobacter rodentium,* among others (www.microbesonline.org). This gene cluster consists of five predicted sugar transporters (YcjN, YcjO, YcjP, YcjV, and OmpG), two sugar dehydrogenases (YcjS and YcjQ), two putative polysaccharide hydrolases/phosphorylases (YcjM and YcjT), an epimerase/isomerase (YcjR), an enzyme that is a presumptive β-D-phosphoglucomutase (YcjU), and a predicted LacI-type repressor (YcjW).

Here we describe our attempts to functionally characterize the substrate profiles for the two putative polysaccharide hydrolase/phosphorylase enzymes (YcjT and YcjM) and YcjU, the presumptive β-D-phosphoglucomutase. The sequence similarity network (SSN) for YcjM (cog0366) at an *E*-value cutoff of 1 x 10^{-50} (Figure 2) shows that the closest functionally characterized homologs include α-(1,2)-D-glucose-D-glycerate phosphorylase (GGP) from

Meiothermus silvanus, sucrose phosphorylase from *Bifidobacteria adolescentis,* and amylosucrase from *Neisseria polysaccharea.*[19–22] For YcjT (cog1554) the SSN at an E-value cutoff of 10^{-150} (Figure 3) indicates that the closest functional homologues of this enzyme include maltose phosphorylase from *Lactobacillus brevis,* trehalose phosphorylase from *Bacillus stearothermophillus*, nigerose phosphorylase from *Lachnoclostridium phytofermantans*, α-(1,2)-D-glucose-glycerol phosphorylase from *Bacillus selenitireducens*, and kojibiose phosphorylase (KBP) from *Thermoanaerobacter brockii* and *Caldicellulosiruptor saccharolyticus*.[23–28] There are two experimentally verified enzymes in the SSN for YcjU (Figure 4): β-D-phosphoglucomutase (PgmB) from *Lactoc0ccus lactis* and β-D-phosphoglucomutase (YvdM) from *Bacillus subtilis* (PDB id: 3NAS).[29] It appears likely that YcjU is a β-D-phosphoglucomutase (β-PGM). However, to the best of our knowledge, there are no currently known enzymes in *E. coli* K-12 that have been shown to catalyze the formation of β-D-glucose-1-P.

MATERIALS and METHODS

Materials

The restriction endonucleases, *pfu* turbo polymerase and T4 DNA ligase used in cloning of the genes *ycjM*, *ycjT*, and *ycjU* from *E. coli* were purchased from New England BioLabs. The PCR cleanup and gel extraction kits were obtained from Qiagen. Isopropyl-β-D-thiogalactopyranoside (IPTG) was acquired from Research Products International Corporation. The buffers, phenylmethylsulfonyl fluoride (PMSF), and the Phosphate Colorimetric Detection Kit were purchased from Sigma Aldrich. The 5-mL HisTrap columns, PD-10 desalting columns, and Vivaspin protein concentrators (molecular weight cut-off of 30 kDa) were bought from GE Healthcare. The M9 minimal media salts and thiamine were obtained from Sigma Aldrich. The carbohydrates used as potential substrates for YcjM, YcjT, and YcjU were obtained from Carbosynth, except for α-D-glucose-1,6-bisphosphate, D-glycerate, and α-D-glucose-1-P, which were obtained from Sigma Aldrich. α-(1,2)-D-Glucose-D-glycerate was synthesized by a modification of a previously published procedure.[30] The following bacterial strains were purchased from the Keio collection: parent strain for the knockouts BW25113 (CGSC 7636), Δ*ycjM* (CGSC 9167), Δ*ycjR* (CGSC 11236) and Δ*ycjT* (CGSC 9173) (https://cgsc2.biology.yale.edu/KeioList.php).

Synthesis of Alternate Substrates for YcjU—2,3,4,5-Tetra-*O*-acetyl-β-mannose was synthesized from D-mannose as previously described.[31] Similarly, 2,3,4,5-tetra-*O*-acetyl-β-D-allose was synthesized from D-allose.[32] 2,3,4,5-Tetra-*O*-acetyl-β-galactose was synthesized from β-D-galactose pentaacetate by modification of a previously published procedure.[33] The phosphorylation of the acetylated sugars and subsequent isolation of β-D-mannose-1-P, β-D-allose-1-P and β-D-galactose-1-P was conducted according to Zhu *et al.*[34]

Construction of Sequence Similarity Networks

Sequence similarity networks were created using Cytoscape (https://cytoscape.org). For YcjT, all members of cog1554 were downloaded from NCBI as a FASTA file. Protein sequences within this file that shared ≥ 90% identity with one another were consolidated.

After this redundancy check was performed using the CD-HIT website, the custom FASTA file was uploaded to the Enzyme Function Initiative (EFI) enzyme similarity tool website (http://efi.igb.illinois.edu/efi-est/stepa.php).[35, 36] The tool-generated data set was analyzed under an organic layout using the Cytoscape software.[37] With YcjM, members of cog0366 were downloaded from the NCBI database to make the Cytoscape network after a redundancy cut-off of 85%. For YcjU (cog0637), members of the Interpro family IPR010972 were used to generate the network for this protein after a redundancy cut-off of 90%.

Cloning of *ycjM* from *E. coli* K-12 MG1655

The following set of primers was used to amplify the DNA corresponding to *ycjM* (gi|90111247; UniProt P76041) from *E. coli* K-12 MG1655 genomic DNA:

5′- AATACTGGATCCATGAAACAGAAAATT -3′

5′- ATAGTACTCGAGTTATTTAATCCACAT -3′

The amplified gene and the pET30a (+) vector were digested with *Bam*HI and *Xho*I restriction enzymes and ligated together to create the recombinant plasmid. After verification of the gene sequence, *E. coli* BL-21 (DE3) cells were transformed with the plasmid and plated on LB agar.

Expression and Purification of YcjM

A single colony was used to inoculate a 10-mL culture of LB, which was grown overnight at 37 °C. The overnight culture was used to initiate two 1-L cultures of LB supplemented with 50 μg/mL kanamycin in 2.8-L Fernbach flasks. The cultures were allowed to grow at 37 °C until the OD_{600} reached ~0.6, the temperature was reduced to 25 °C, and the cultures allowed to grow overnight without the addition of IPTG. The cells were harvested by centrifugation at 11,000 g for 12 min and the cell pellet was stored at -80 °C until needed.

The frozen cell pellet was thawed and suspended in 20 mM HEPES/K^+, 0.5 M KCl, pH 8.0 at 4 °C. Prior to sonication, 0.1 mg/mL PMSF and 0.4 mg/mL DNase I were added to the cell suspension and stirred for 15 min. The cells were lysed with a Branson Sonifier 450 for six 5-minute intervals at 50% output. After sonication, the cell suspension was clarified by centrifugation at 12,000 g for 20 min. The supernatant fluid was passed through a 0.45 μm syringe filter, and loaded onto a 5-mL HisTrap column at room temperature, which had previously been equilibrated with binding buffer. The protein of interest was eluted from the column by applying a gradient of the elution buffer (20 mM HEPES/K^+, 0.25 M KCl, 0.5 M imidazole, pH 8.0). The isolated protein was >95% pure based on SDS-PAGE. The fractions were collected and concentrated using Vivaspin protein concentrators. The concentrated protein was then passed through a PD-10 desalting column to remove imidazole. Protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 94,700 M^{-1} cm^{-1} and a molecular weight of 69.6 kDa that includes the 5-kDa linker between the His-tag and the protein (https://web.expasy.org/protparam/). The protein was flash-frozen and stored at −80 °C until needed. Approximately 5 mg of the purified enzyme was obtained from 1.0 L of bacterial cell culture.

Determination of Phosphorylase and Hydrolase Activities of YcjM

A small library of α-D-glucose derivatives was tested as potential substrates with YcjM (Scheme S1). The assays for the α-D-glucose-glucose disaccharides contained the following: 1.0 mM disaccharide, 10 mM inorganic phosphate, 5.0 mM $NADP^+$, 2.0 mM ATP, 5.0 mM $MgCl_2$, 1 U hexokinase, 3 U glucose-6-phosphate dehydrogenase (G6PDH), and 2.0 μM YcjM in 50 mM HEPES/K^+, pH 8.0. The α-D-glucose-fructose, α-D-glucose-galactose, and α-D-glucose-glycerate disaccharides were assayed in the presence of 1.0 U α-PGM, 3 U G6PDH, 1.0 mM disaccharide, 10 mM inorganic phosphate, 4.0 mM $NADP^+$, 100 μM α-D-glucose-1,6-bisphosphate, and 2.0 μM YcjM in 50 mM HEPES/K^+, pH 8.0. The assays were repeated with 2.0 μM YcjU (β-PGM) instead of α-PGM.

The assays used to screen the reverse phosphorylase activity of YcjM were conducted at 30 °C using either 50 mM HEPES/K^+, pH 8.0, or 50 mM cacodylate, pH 6.5. The reactions contained 1.0 mM monosaccharide (Schemes S2, S3, and S4), 1.0 mM α-D-glucose-1-P, and 1.0 μM YcjM. Aliquots were removed every hour and the formation of inorganic phosphate was determined using the phosphate detection kit.

The kinetic constants for the phosphorolysis of α-(1,2)-D-glucose-D-glycerate catalyzed by YcjM were measured with varying concentrations (0–350 μM) of α-(1,2)-D-glucose-D-glycerate, 10 mM inorganic phosphate, 0.25 μM YcjM, 4 U α-PGM (activated with 0.25 mM α-D-glucose-1,6-bisphosphate), 8 U G6PDH, 5 mM $MgCl_2$, and 5 mM $NADP^+$ in 50 mM cacodylate/K^+, pH 6.5. Reactions with varying concentrations of inorganic phosphate (0–15 mM) were conducted by keeping the concentration of α-(1,2)-D-glucose-D-glycerate constant at 150 μM. The assays used for measuring the kinetic constants of the reverse reaction contained α-D-glucose-1-phosphate (0–20 mM), 4.0 mM D-glycerate, and 2.5 nM YcjM in 50 cacodylate/K^+, pH 6.5. The reactions were incubated at 30 °C and aliquots were removed every 10 min over a period of 60 min. The concentration of inorganic phosphate was determined using the phosphate detection kit.

Cloning of *ycjT* from *E. coli* K-12 MG1655

The gene for *ycjT* (gi|16129277; UniProt P77154) was amplified from the genomic DNA of *E. coli* K-12 MG1655 using the following set of primers:

5′-AATGCGGATCCATGACCAGGCCAGTAACG- 3′

5′-ATAGTCTCGAGTCATTCATCCTCCTGATGTTTGG -3′

The restriction enzymes *Bam*HI and *Xho*I were used to digest the amplified gene, followed by ligation into a pET30a(+) vector, which was also digested with the same restriction enzymes and purified using the gel extraction kit. After verification of the amplified gene sequence, *E. coli* BL-21 (DE3) cells were transformed with the recombinant plasmid by electroporation.

Expression and Purification of YcjT

A single colony was grown overnight in 20 mL of LB containing 50 μg/mL kanamycin at 37 °C. The overnight culture was divided to inoculate 1 L of LB media supplemented with 50 μg/mL kanamycin in four 2.8-L Fernbach flasks. The cell cultures were grown at 37 °C

until an OD_{600} of ~0.6 was reached. Expression was induced with 0.5 mM IPTG and the cell cultures were allowed to grow overnight at a reduced temperature of 25 °C. The cells were harvested by centrifugation at 11,000 g for 12 min and the cell pellet was stored at −80 °C until needed.

The frozen cell pellet was re-suspended in 50 mM HEPES/K^+, 100 mM KCl, 10 mM imidazole, at pH 7.5. Prior to sonication, 0.1 mg/mL PMSF and 0.4 mg/mL of DNase I were added to the re-suspended cells, and the cell suspension was stirred for 20 min at 4 °C. The cells were lysed by sonication with a Branson Sonifier 450 using three 4-minute intervals at 50% output. After sonication, the cell debris was separated from the soluble proteins in the supernatant solution by centrifugation at 12,000 g for 20 min. The supernatant solution was loaded onto a 5-mL HisTrap column, which had previously been equilibrated with binding buffer. YcjT was eluted from the column by applying a gradient of 50 mM HEPES/K^+, 100 mM KCl, and 500 mM imidazole at pH 7.5. The fractions were analyzed for purity using SDS-PAGE, and the fractions that were judged to be greater than ~90% pure were pooled and dialyzed against a solution of 50 mM HEPES/K^+, pH 7.5, and 100 mM KCl, to remove the imidazole. Protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 145,000 M^{-1} cm^{-1} and molecular weight of 90 kDa, including the 5-kDa linker between the His-tag and the protein (https://web.expasy.org/protparam/). Aliquots of YcjT were flash-frozen and stored at −80 °C until needed. Approximately 25 mg of YcjT was obtained from 1.0 L of bacterial cell culture.

Determination of Phosphorylase and Hydrolase Activity of YcjT

All kinetic assays were carried out at 30 °C in 50 mM HEPES/K^+, 100 mM KCl, at pH 7.5. For potential substrates consisting of a glucose/glucose disaccharide (Scheme S1), 1.0 mM of the disaccharide, 10 mM inorganic phosphate, 5.0 mM $MgCl_2$, 2.0 mM ATP, 5.0 mM $NADP^+$, and 0.5 μM YcjT was mixed with 1 U hexokinase and 3 U G6PDH in a total volume of 250 μL. The increase in absorbance was monitored at 340 nm ($\Delta\varepsilon$ = 6220 M^{-1} cm^{-1}). To determine the potential catalytic activity with glucose-galactose or glucose-fructose disaccharides (Scheme S1), the reaction mixtures contained 1.0 mM disaccharide, 10 mM inorganic phosphate, 5.0 mM $MgCl_2$, 2.0 mM ATP, 5.0 mM $NADP^+$, 1.0 μM YcjU, 3 U of G6PDH, and 0.5 μM YcjT in a total volume of 250 μL. The formation of NADPH was measured at 340 nm.

Assays designed to identify potential substrates for the reverse phosphorolysis reaction catalyzed by YcjT contained 1.0 mM monosaccharide (Schemes S2, S3, and S4), 250 μM β-D-glucose-1-phosphate, 50 mM HEPES/K^+, 100 mM KCl, pH 7.5, and 0.5 μM YcjT. The reactions were incubated at room temperature, and aliquots were removed every 30 min for a total of 4 h and the release of inorganic phosphate was measured using the phosphate detection kit.

The kinetic constants for the phosphorolysis of D-kojibiose by YcjT were conducted in 50 mM HEPES/K^+ and 100 mM KCl at pH 7.5 using an assay containing (0 – 15) mM kojibiose, 15 mM inorganic phosphate, 5.0 mM $MgCl_2$, 5.0 mM $NADP^+$, 1.0 μM YcjU, 3 U G6PDH, and 0.5 μM YcjT in a total volume of 250 μL. To determine the kinetic constants for phosphate, the assays were conducted with 5.0 mM kojibiose, 0.5 μM YcjT, and various

concentrations (0 – 25 mM) of inorganic phosphate in the presence of 5.0 mM $MgCl_2$, 5.0 mM ATP, 5.0 mM $NADP^+$, 2 U hexokinase, 3 U G6PDH in 50 mM HEPES/K^+, 100 mM KCl, pH 7.5 at 30 °C. The initial velocity was calculated by monitoring the formation of NADPH at 340 nm.

To determine if YcjT hydrolyzed β-D-mannose-1-P, β-D-allose-1-P, and β-D-galactose-1-P, the reactions were conducted in the presence of 1.0 mM sugar phosphate and 1.0 μM YcjT in 50 mM HEPES/K+, pH 7.5, at 30 °C. The reactions were assayed for the liberation of phosphate every 30 min for 3 h with the phosphate detection kit.

Cloning of *ycjU* from *E. coli* K-12 MG1655

The gene *ycjU* (gi|16129278; UniProt P77366) was amplified from the genomic DNA of *E. coli* K-12 MG1655 using the following set of primers:

5′-AATGCGGATCCATGAAACTGCAAGGGGTAATTTTCGATCTGG-3′

5′-ATAGTCTCGAGCTATACGTTTTGCCAGAAGGCCGATAACC-3′

The amplified gene was purified using the PCR cleanup kit and digested with the restriction enzymes *Bam*HI and *Xho*I. The digested gene was ligated into a pET30a(+) vector, which was previously digested with the same two restriction enzymes and purified using the gel extraction kit. After verification of the gene sequence, *E. coli* BL-21 (DE3) cells were transformed with the plasmid and plated on LB agar.

Expression and Purification of YcjU

A single colony was used to initiate a 10-mL culture of LB, which was grown overnight at 37 °C. The overnight culture was used to inoculate two 1.0-L cultures of LB supplemented with 50 μg/mL kanamycin and 1.0 mM $MgCl_2$ in 2.8-L Fernbach flasks and then allowed to grow at 30 °C until the OD_{600} reached ~0.6. Protein overexpression was induced with 0.5 mM IPTG and the cell cultures were grown overnight at a temperature of 25 °C. The cells were harvested by centrifugation at 11,000 g for 12 min and the cell pellet was stored at −80 °C until needed for the purification of YcjU.

The frozen cell pellet was re-suspended in a binding buffer containing 20 mM HEPES/K^+, 0.25 M KCl, and 15 mM imidazole at pH 7.9 at 4 °C. Prior to sonication, 0.1 mg/mL PMSF and 0.4 mg/mL of DNase I were added to the re-suspended cells and then stirred for 15 min. The cells were lysed with a Branson Sonifier 450 for three 4-min intervals at 50% output. After sonication, the cell debris was separated from the soluble proteins in the supernatant solution by centrifugation at 12,000 g for 20 min. The supernatant solution was passed through a 0.2 μm syringe filter, and loaded onto a 5-mL HisTrap column at room temperature, which had previously been equilibrated with binding buffer. YcjU was eluted from the column by applying a gradient of 20 mM HEPES/K^+, 0.25 M KCl, and 500 mM imidazole, pH 7.9. The column fractions were analyzed for purity using SDS-PAGE, and fractions with more than 90% purity were pooled and dialyzed against a solution of 20 mM HEPES/K^+ and 100 mM KCl at pH 8.0 to remove the imidazole. Protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 19,500 M^{-1} cm^{-1} and molecular weight of 28.5 kDa that includes the 5-kDa linker between the His-tag

and the protein (https://web.expasy.org/protparam/). The protein was flash-frozen and stored at −80 °C until needed. Approximately 35 mg of purified enzyme was obtained from 1.0 L of bacterial cell culture.

Determination of YcjU Activity

The kinetic assays were conducted at 30 °C in 50 mM HEPES/K^+, pH 8.0. The formation of D-glucose-6-phosphate from β-D-glucose-1-phosphate was determined by measuring the increase in absorbance at 340 nm from the G6PDH catalyzed reduction of $NADP^+$. The assays were carried out in the presence of varying amounts (0 – 10 mM) of β-D-glucose-1-P using 100 μM of α-D-glucose-1,6-bisphosphate as the activator, 0.1 μM YcjU, 2.0 units of G6PDH, 2.0 mM $NADP^+$, and 2.0 mM $MgCl_2$.

To determine the equilibrium constant of the YcjU catalyzed reaction, ^{31}P NMR (161 MHz, relaxation delay of 1.0 sec) was used to measure the relative concentration of D-glucose-6-P and β-D-glucose-1-P. The forward reaction contained 2.0 mM β-D-glucose-1-P, 125 μM α-D-glucose-1,6-bisphosphate, 1.0 mM $MgCl_2$, and 0.1 μM YcjU in 50 mM HEPES/K^+, 100 mM KCl, pH 8.0 with 10% D_2O. After incubation with the enzyme for 1.0 h, 2.0 mM EDTA was added to quench the reaction. The reverse reaction was conducted in a similar manner with the exception of adding 2.0 mM D-glucose-6-P instead of β-D-glucose-1-P to initiate the reaction.

^{31}P-NMR experiments were used to determine the activity of YcjU with β-D-mannose-1-P, β-D-allose-1-P, and β-D-galactose-1-P. The reaction mixtures contained 3.0 mM sugar phosphate, 100 μM α-D-glucose-1,6-bisphosphate as an activator, and 2.0 mM $MgCl_2$ in 50 mM HEPES/K^+, 100 mM KCl, pH 8.0. Concentration of YcjU used to initiate the reaction with β-D-mannose-1-P and β-D-allose-1-P was 2.5 μM and 0.05 μM respectively. ^{31}P NMR spectra were recorded before and after addition of YcjU. Scans were taken every 30 min after addition of YcjU for a total period of 2 h. Integration of the NMR resonances for the substrate and product was used to monitor the change in substrate and product concentrations as a function of time. The equilibrium constants were determined using an initial concentration of either 2.0 mM of the sugar-1-P substrate or 2.0 mM of the corresponding sugar-6-P product. The reactions contained 100 μM α-D-glucose-1,6-bisphosphate, 1.0 mM $MgCl_2$, and 1.0 μM YcjU in 50 mM HEPES/K^+, 100 mM KCl, pH 8.0 and were allowed to proceed for 3–6 h to reach equilibrium. The reactions were quenched by the addition of 3 mM EDTA.

Determination of YcjU Activation by Mg^{2+}

To determine the effect of added Mg^{2+} on the catalytic activity of YcjU, assays were initiated with 100 μM β-D-glucose-1-P, 2.0 mM $NADP^+$, 50 μM α-D-glucose-1,6-bisphosphate, 0.2 μM YcjU, 3 U G6PDH, and varying amounts (0 - 10 mM) of Mg^{2+} in 50 mM HEPES/K^+, 100 mM KCl, pH 8.0 at 30 °C. The initial velocity associated with D-glucose-6-phosphate formation was determined by monitoring the increase in absorbance at 340 nm.

Determination of Kinetic Constants

The values of k_{cat} and k_{cat}/K_m were determined from the initial velocity data using Eqn. 1 where v is the velocity, E_t is the enzyme concentration, A is the substrate concentration, k_{cat} is the turnover number, and K_m is the Michaelis constant.

$$v/E_t = k_{cat}\,A/(K_m + A) \quad (1)$$

Growth of *E. coli* on Various Carbon Sources

M9 minimal media was prepared from commercially available M9 minimal media salts (Na_2HPO_4, KH_2PO_4, NH_4Cl, and NaCl). Minimal media plates were prepared from minimal media, 1.5% (w/v) of agar with 100 μM thiamine, and 0.25% w/v carbon source (D-glucose or D-kojibiose) added to the autoclaved media before plating. Single colonies of BW25113, Δ*ycjT*, and Δ*ycjM* were grown in 5 mL of LB media overnight at 37 °C. BL-21 (DE3) cells, transformed with the *ycjT* overexpression plasmid, were grown in 5 mL of LB at 37 °C until an OD_{600} reached ~0.6. The culture was induced with 0.5 mM IPTG and allowed to grow overnight at 25°C. From each of the 5-mL cultures, 1 mL was removed, centrifuged at 16,000 g for 2 min, and the cell pellets re-suspended in minimal media. The cell suspensions were centrifuged at 16,000 g for 2 min followed by re-suspension in minimal media. This 'washing' was repeated five times to ensure that no LB media was carried over with the cells onto the minimal media plates. In order to determine the number of colonies associated with different dilution factors for the different bacterial strains, the cell suspensions were serially diluted (up to 10^{-6}) and each serial dilution was plated on minimal media plates with either 0.25% w/v glucose or kojibiose. Plates with no added carbon source were used as negative control plates. The plates with BL-21 (DE3) also had 0.25 mM IPTG added to maintain overexpression levels of YcjT. SDS-PAGE was conducted with the BL-21(DE3) cell culture to confirm overexpression of YcjT before plating. Growth on the plates was monitored for a total of 4 days. To determine growth of different strains of bacteria on various glucose-glucose disaccharides, BW25113 and BL-21 (DE3) were plated on minimal media plates supplemented with 0.5% (w/v) of α,α-trehalose, kojibiose, sophorose, laminaribiose, nigerose, maltose, cellobiose, isomaltose, and gentiobiose as potential carbon sources. Minimal media plates with various concentrations (0.1% w/v, 0.25% w/v, and 0.5% w/v) of kojibiose, glucose, succinate, and sucrose were also made to test potential carbon sources for BW25113 strain.

RESULTS

Catalytic Activity of YcjM

YcjM was purified to homogeneity and subsequently tested as a catalyst for the hydrolysis or phosphorolysis of the library of disaccharides presented in Scheme S1. Based on the Cytoscape network constructed for YcjM (Figure 2), α-(1,2)-D-glucose-D-glycerate phosphorylase and sucrose phosphorylase are the two closest experimentally verified enzymatic functions for enzymes in cog0366. Of the compounds tested, YcjM exhibited catalytic activity only with α-(1,2)-D-glucose-D-glycerate (**1**) in the presence of phosphate.

The two products were determined to be α-D-glucose-1-P (**2**) and D-glycerate (**3**), and the overall reaction is summarized in Scheme 1a. YcjM was unable to catalyze the hydrolysis of any of the other compounds listed in Scheme S1. The kinetic constants for the phosphorolysis of α-(1,2)-D-glucose-D-glycerate are as follows: k_{cat} = 2.1 s^{-1}, K_m = 69 μM, and k_{cat}/K_m = 3.1 x 10^4 M^{-1} s^{-1} at a fixed concentration of 20 mM phosphate at pH 6.5. The K_m for phosphate was determined to be 2.2 mM in the presence of 1.0 mM α-(1,2)-D-glucose-D-glycerate and varying (0 – 20 mM) concentrations of inorganic phosphate.

YcjM catalyzed the hydrolysis of α-D-glucose-1-P with a k_{cat} = 2.4 s^{-1}, K_m = 15.2 mM, and k_{cat}/K_m = 1.6 x 10^2 M^{-1} s^{-1}. Less than 1% of this activity was detected in the presence of α-D-galactose-1-P, α-D-mannose-1-P or β-D-glucose-1-P. A small library of monosaccharides (Schemes S2, S3, and S4) was tested as potential acceptor substrates with α-D-glucose-1-P in the back reaction. Of the compounds tested, catalytic activity was observed with D-glycerate (**3**) and D-erythronate. At a fixed concentration of 4.0 mM D-glycerate, the apparent kinetic parameters for α-D-glucose-1-P (**2**) during the formation of α-(1,2)-D-glucose-D-glycerate are k_{cat} = 350 s^{-1}, K_m = 9.4 mM, and k_{cat}/K_m = 3.7 x 10^4 M^{-1} s^{-1}. In the presence of 10 mM α-D-glucose-1-P, the K_m for D-glycerate was determined to be 4.4 mM. At a fixed concentration of 10 mM α-D-glucose-1-P and varying concentrations (0–20 mM) of D-erythronate, the kinetic parameters of the enzymatic reactions were k_{cat} = 68 s^{-1}, K_m = 8 mM, and k_{cat}/K_m = 8.5 x 10^3 M^{-1} s^{-1}. Less than 5% of this activity was observed with either L-glycerate, D-ribonate, D-allonate or glycerol. The kinetic constants for the reactions catalyzed by YcjM are summarized in Table 1.

Catalytic Activity of YcjU

Based on the available information from the sequence similarity network diagram (Figure 3) the proposed catalytic properties of YcjU as a β-phosphoglucomutase were tested with β-D-glucose-1-P (**4**) (Scheme 1b). To fully activate YcjU by phosphorylation, the enzyme was incubated with 100 μM α-D-glucose-1,6-bisphosphate for 15 min prior to the kinetic assays. In the β-phosphoglucomutase from *Lactococcus lactis*, the addition of α-D-glucose-1,6-bisphosphate to the enzyme facilitates the transfer of the phosphoryl group at C6 to Asp-8 in the active site. The phosphoryl group is subsequently transferred to the substrate β-D-glucose-1-P to form the β-D-glucose-1,6-bisphosphate intermediate.[38, 39] We assume that the activation of YcjU by α-D-glucose-1,6-bisphosphate operates in a similar fashion. The kinetic parameters for the interconversion of β-D-glucose-1-P to D-glucose-6-P are as follows: k_{cat} = 21 s^{-1}, K_m = 18.5 μM, and k_{cat}/K_m = 1.1 x 10^6 M^{-1} s^{-1}. Substrate inhibition was observed at concentrations of β-D-glucose-1-P greater than ~0.2 mM. When the activity of YcjU was tested in the presence of varying concentrations of Mg^{2+} (0 – 10 mM) with 0.1 mM β-D-glucose-1-P, the activation constant (K_{act}) for Mg^{2+} was determined to be 135 ± 2 μM. In the absence of added Mg^{2+} the observed catalytic activity was less than 2% of the turnover observed in the presence of 2.0 mM Mg^{2+}. YcjU exhibited <1% of the catalytic activity (relative to β-D-glucose-1-P) with α-D-glucose-1-P. When YcjU was not phosphorylated with α-D-glucose-1,6-bisphosphate, less than 5% of the enzyme activity was observed. In the reverse direction, the kinetic parameters for the formation of β-D-glucose-1-P from D-glucose-6-P are as follows: k_{cat} = 0.5 s^{-1}, K_m = 1.19 mM, and k_{cat}/K_m = 4.2 x 10^2 M^{-1} s^{-1}. The kinetic constants for the reactions catalyzed by YcjU are

summarized in Table 1. The equilibrium constant for the YcjU-catalyzed reaction was calculated to be 47 ± 2 from an integration of the ^{31}P NMR resonances for β-D-glucose-1-P and D-glucose-6-P when YcjU was added to either substrate and allowed to reach equilibrium.

The YcjU catalyzed reactions with β-D-allose-1-P, β-D-galactose-1-P, and β-D-mannose-1-P are shown in Scheme S5. In the presence of saturating concentrations of β-D-allose-1-P, β-D-galactose-1-P, and β-D-mannose-1-P, apparent k_{cat} was calculated to be 10.5 ± 0.2 s^{-1}, 0.47 ± 0.09 s^{-1} and 0.09 ± 0.01 s^{-1} respectively. The equilibrium constants for the YcjU catalyzed reactions with β-D-allose-1-P, β-D-galactose-1-P and β-D-mannose-1-P were 24 ± 3, 29 ± 2 and 30 ± 3 respectively.

Catalytic Activity of YcjT

Based on the reported substrates for the closest functional homologs of YcjT from the sequence similarity network presented in Figure 4, a broad list of potential disaccharides was tested for catalytic activity. Among all of the disaccharides that were tested for catalytic activity (Scheme S1), YcjT showed phosphorylase activity only with D-kojibiose (Scheme 1c). The remaining disaccharides were less than 1% as active as D-kojibiose (**6**) at a concentration of 1.0 mM. At a fixed concentration of 5.0 mM phosphate, the apparent kinetic parameters for the phosphorolysis of D-kojibiose (0–15 mM) are as follows: k_{cat} = 1.1 s^{-1}, K_m = 1.05 mM, and k_{cat}/Km = 1.1 x 10^3 M^{-1} s^{-1}. In the presence of varying concentrations of phosphate (0–25 mM) at a fixed concentration of 5.0 mM kojibiose, the K_m for phosphate was determined to be 3.0 mM.

To determine the identity of the phosphorylated product from the phosphorolysis of D-kojibiose, the catalytic activity of YcjT was coupled to either α-phosphoglucomutase or YcjU (β-PGM) and G6PDH. Activity was observed only when the product of the reaction was coupled with YcjU, indicating that the other product (in addition to D-glucose) is β-D-glucose-1-P.

The catalytic properties of the reverse reaction were determined by incubating YcjT with β-D-glucose-1-P and various monosaccharides (Schemes S2, S3, and S4). Catalytic activity (measured by the formation of phosphate) was observed only with D-glucose, L-sorbose, D-sorbitol, L-iditol or 1,5-anhydro-D-glucitol, and the proposed structures of the corresponding disaccharides (compounds **6**, **9–12**) formed in these reactions are shown in Scheme 2. The rate constants for the hydrolysis of β-D-glucose-1-P in the absence of an acceptor substrate were determined to be k_{cat} = 0.014 s^{-1}, K_m = 2.01 mM and k_{cat}/K_m = 7.0 M^{-1} s^{-1}. At a fixed concentration of 8.0 mM β-D-glucose-1-phosphate, the apparent kinetic parameters for the formation of D-kojibiose (k_{cat} = 0.8 s^{-1}, K_m = 1.7 mM, and k_{cat}/K_m = 2.6 x 10^2 M^{-1} s^{-1}), α-(1,2)-D-glucose-anhydro-D-glucitol (**12**; k_{cat} = 1.05 s^{-1}, K_m = 6.1 mM, and k_{cat}/K_m = 1.7 x 10^2 M^{-1} s^{-1}), α-(1,5)-D-glucose-L-sorbose (**9**; k_{cat} = 1.2 s^{-1}, K_m = 7.9 mM, and k_{cat}/K_m = 1.5 x 10^2 M^{-1} s^{-1}), α-(1,2)-glucose-D-sorbitol (**10**; k_{cat} = 1.2 s^{-1}, K_m = 12 mM, and k_{cat}/K_m = 1.0 x 10^2 M^{-1} s^{-1}), and α-(1,2)-D-glucose-L-iditol (**11**; k_{cat} = 1.3 s^{-1}, K_m = 14 mM, and k_{cat}/Km = 92 M^{-1} s^{-1}) were determined. In an attempt to determine if YcjT can synthesize higher order oligosaccharides with β-D-glucose-1-P, various glucose-glucose disaccharides (Scheme S1) were tested. None of the disaccharides showed any

significant activity (< 1%) with YcjT when compared to the rate with D-glucose. YcjT did not show any phosphate release when β-D-mannose-1-P, β-D-galactose-1-P, and β-D-allose-1-P were incubated with the enzyme in the presence of D-glucose. The kinetic constants for the reactions catalyzed by YcjT are summarized in Table 1.

Growth on Kojibiose

To determine the ability of kojibiose to function as the sole carbon source for *E. coli*, overnight cultures of various cell lines (BW25113, BW25113 Δ*ycjT*, BW25113 Δ*ycjM,* and BL21 (DE3) with pET30a (+) containing *ycjT*) were washed, serial diluted, and then plated on minimal media with either D-kojibiose or D-glucose. Colony forming units were observed for all strains on glucose plates with 100 μL of the 10^{-6} dilution culture. No colonies were observed on any of the plates in the absence of a carbon source (negative control). None of the bacterial strains grew on the plates supplemented with kojibiose. Of the various disaccharides tested as potential carbon sources for BW25113 and BL-21 (DE3), only α,α-trehalose and maltose supported growth. No growth was observed on plates with kojibiose, sophorose, laminaribiose, nigerose, cellobiose, isomaltose or gentiobiose as the sole carbon source.

DISCUSSION

There are numerous enzymes that are responsible for the construction and disassembly of complex carbohydrate structures. Given the vast diversity in carbohydrate structures and the specificity of enzymes for their respective substrates, characterization of these enzymes has been challenging.[40] Glycoside phosphorylases have garnered interest from the biotechnology industry because of their potential in synthesizing unusual glycosides that have commercial applications in the cosmetic industry, and as food additives.[41–44] Genomic analysis of bacteria that inhabit the human gut show that numerous enzymes are annotated as carbohydrate-active enzymes but a large fraction of these proteins have not been experimentally interrogated.[45] Here we describe our efforts to characterize three enzymes (YcjM, YcjT, and YcjU) from a putative carbohydrate metabolic pathway that is present in a variety of Gram-negative bacteria in the human gut, including various strains of *E. coli.*

Functional Characterization of YcjM, a D-Glucose-D-Glycerate Phosphorylase

YcjM belongs to cog0366 and shares 46% identity with glucose-glycerate phosphorylase from *Meiothermus silvanus*, and 27% identity with sucrose phosphorylase from *Bifidobacterium adolescentis* (PDB: 1R7A) and amylosucrase from *Neisseria polysaccharea* (PDB: 1JGI).[20–22] All four enzymes belong to the retaining glycoside hydrolase family 13 (GH13), also known as the α-amylase family, according to the CAZy database.[46] Members of this enzyme family catalyze reactions through a double displacement mechanism that proceeds though a covalent glucose-enzyme intermediate and involves a nucleophilic aspartate residue and a glutamate residue which acts as a proton donor.[47]

A homology model of YcjM was built using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) with sucrose phosphorylase (PDB id: 2GDU) as the template. A

structural comparison between sucrose phosphorylase (PDB id: 2GDU), amylosucrase (PDB id: 1JGI) and the YcjM model was assessed using Pymol. Homology modeling of D-glucose-D-glycerate phosphorylase from *M. silvanus,* also built with sucrose phosphorylase as the structural template, followed by docking of α-(1,2)-D-glucose-D-glycerate into the active site showed that Asn275 and Glu383 appeared to be positioned to interact productively with D-glycerate.[20] Both of the homologous residues in YcjM (Asn273 and Glu382) are conserved (Figure S1). In the YcjM model, the docked α-(1,2)-D-glucose-D-glycerate superimposes well with the bound sucrose when overlaid with the sucrose phosphorylase structure (PDB id: 2GDU) from *B. adolescentis*. The catalytic residues, Asp229 and Glu271, are 4.4 Å and 4.3 Å from the anomeric carbon, respectively. Based 39on this model, Arg443 can potentially interact with the carboxylate group of α-(1,2)-D-glucose-D-glycerate. There are two other arginine residues in the vicinity (Arg171 and Arg378) that can potentially move in closer to the bound substrate to provide additional electrostatic interactions.

α-(1,2)-D-Glucose-D-glycerate, once considered a rare osmoprotectant, is now known to accumulate under conditions of salt stress and nitrogen scarcity in enterobacteria, marine cyanobacteria, and halophilic methanogens.[48–51] Two species of *Streptococcus* have been reported to accumulate D-glucose-D-glycerate, along with trehalose.[52, 53] *Streptococcus caelestis* is also known to excrete D-glucose-D-glycerate into the medium.[52] Glucose-D-glycerate was first identified in the 1960s as part of the reducing ends of the methylglucose lipopolysaccharide (MGLP) in *Mycobacterium phlei*.[54]

There are two common pathways for the biosynthesis of D-glucose-D-glycerate. The first one involves a two-step biosynthetic route using glucose-3-phosphoglycerate synthase and glucose-3-phosphoglycerate phosphatase.[55] The physiological role of MGLP in the formation of mycolic acids has been suggested and since the deletion of glucose-3-phosphoglycerate synthase was shown to affect the biosynthesis of MGLP, it is considered as a potential anti-tuberculosis drug target.[56, 57] The second route is catalyzed by glucose-glycerate synthase which converts D-glycerate and ADP-glucose to D-glucose-D-glycerate and ADP.[58]

Functional Characterization of YcjT, a Kojibiose Phosphorylase

Kojibiose phosphorylase belongs to the inverting glycoside hydrolase family 65 (GH65), which also includes trehalose phosphorylase and maltose phosphorylase.[59] The product of this reaction is β-D-glucose-1-P, which is the apparent substrate for the next enzyme in the pathway, YcjU. YcjT, which belongs to cog1554, shares 30% sequence identity with orthologs from *Thermoanaerobacter brockii*, *Caldicellulosiruptor saccharolyticus*, and an archaea, *Pyrococcus sp.*.[27, 28, 60] Kojibiose phosphorylases have previously been shown to transfer β-glucose-1-P to alternative acceptor substrates, apart from glucose. Our studies showed that among the monosaccharides tested in presence of β-D-glucose-1-P, only 1,5-anhydro-D-glucitol, L-sorbose, D-sorbitol or L-iditol can function as alternative substrates in the back reaction. The formation of a disaccharide with L-sorbose, D-sorbitol, glycerol, and *myo*-inositol has been reported previously with kojibiose phosphorylase from *T. brockii.*[27, 61] As the stereochemistry of the hydroxyl groups at C5, C4, and C3 of L-sorbose

correspond to the hydroxyl groups at C2, C3, and C4 of D-glucose, the glycoside bond of the D-glucose-L-sorbose disaccharide is 1,5 rather than 1,2. YcjT did not exhibit significant catalytic activity for the formation of larger oligosaccharides when incubated with β-D-glucose-1-P and kojibiose. On the contrary, the kojibiose phosphorylase from *C. saccharolyticus* prefers kojitriose as an acceptor relative to kojibiose.[28]

Based on the multiple sequence alignment (Figure S2) between YcjT, and kojibiose phosphorylases from *C. saccharolyticus* (PDB id: 3WIQ), *T. brockii*, and *P. horikoshii*, the residues involved in the binding of kojibiose (Tyr327, Asp334, Trp333, Trp381, Glu382, Lys573, Gln574, Ser608, and Ser609) are conserved with the exception of the presence of serine (Ser409) in YcjT as compared to threonine in kojibiose phosphorylases from the other three organisms.[62] The proposed general acid residue, Glu473 is also conserved in YcjT.

Attempts to grow BW25113, and deletions strains of Δ*ycjT* and Δ*ycjM* on kojibiose were unsuccessful. BL-21 (DE3) with a YcjT overexpression plasmid also failed to grow on kojibiose in the presence of 0.5 mM IPTG. This result suggests that kojibiose is probably not transported inside the cells. It appears that the *ycj* pathway, in addition to the putative ABC-type transporter complex (YcjNOP), has OmpG, which is a large, monomeric porin protein (PDB id: 2IWW) and is believed to be involved in the unspecific uptake of oligosaccharides.[63] All attempts to express this protein under laboratory conditions have been unsuccessful.[64] Our experiments indicate that kojibiose is most likely not the inducer for the *ycj* pathway.

Functional Characterization of YcjU, a β-Phosphoglucomutase

An InterPro search with the protein sequence shows that YcjU belongs to the HAD hydrolase superfamily, subfamily IA and cog0637. In *E. coli*, the source of α-glucose-1-phosphate is primarily from the breakdown of glycogen by glycogen phosphorylase.[65] The catalytic activity of kojibiose phosphorylase releases β-glucose-1-P in *E .coli,* which is a substrate for YcjU. The catalytic efficiencies of β-phosphoglucomutase (β-PGM) from *Lactococcus lactis* and YcjU are comparable.[66] The two enzymes share 44% identity and a comparison of their structures *L. lactis* β-PGM (PDB id: 1LVH) and *E. coli* YcjU (PDB id: 4G9B) show that the active site residues are mostly conserved. YcjU exhibits catalytic activity with β-D-mannose-1-P, β-D-allose-1-P and β-D-galactose-1-P to form the corresponding sugar-6-P product. This activity has not previously been reported before, but these β-sugar-1-P products are not substrates for the back reaction catalyzed by YcjT as a replacement for β-D-glucose-1-P. A derivative of *Escherichia coli* K-12 with a mutation in *ycjU* has been reported to be UV-radiation sensitive showing that this enzyme may have other physiological roles in facilitating survival of *E. coli* cells against agents that damage DNA.[67]

The gene cluster responsible for the metabolism of kojibiose in *C. saccharolyticus, T. brockii* , and *Pyrococcus sp.* consists of genes encoding for sugar transport proteins, a transcription regulator, kojibiose phosphorylase, β-phosphoglucomutase and an additional phosphorylase, which also belongs to cog1554. The *ycj* gene cluster in *E. coli*, and in other organisms where this pathway is conserved, has a few additional genes that encode for proteins with the following putative functions: YcjQ and YcjS are NAD^+-dependent sugar

dehydrogenases, YcjR, an epimerase/isomerase, and OmpG, a porin.[68] This observation indicates that the fate of the breakdown products of kojibiose in these organisms might be different from that of *C. saccharolyticus*, *T. brockii*, and *Pyrococcus* sp.

Kojibiose is a rare sugar, which is present in low levels in sake and koji extracts, honey, and beer.[69–71] In the human gut, kojibiose is also found as part of the 'intracellular' teichoic acids in Gram-positive bacteria such as *Streptococci*.[72] Studies have uncovered that these teichoic acids may have a role to play in conferring resistance to β-lactams in Methicillin-resistant *Staphylococcus aureus* (MRSA) and the biogenesis now serves as a target for drug design.[73] Kojibiose has recently gained commercial interest as a potential low calorie sweetener because α-(1,2)-glycosidic bonds are apparently resistant to breakdown by enzymes in the digestive tract.[74–77] Moreover, kojibiose has a prebiotic effect on beneficial colonic bacterial species such as *Bifidobacteria* and *Lactobacillus* in the gut with a high prebiotic index of 22 making it an ideal candidate for an alternate sugar substitute.[74, 77, 78] Additionally, kojibiose has shown inhibitory effects on α-glucosidase I in rat liver microsomes, yeast microsomal extracts, and bovine mammary glands.[79–81] This observation has unveiled the potential for using this disaccharide as part of the pseudo disaccharide class of drugs that targets human immunodeficiency virus (HIV) as well as inhibiting intestinal α-glucosidases as a potential treatment for diabetes mellitus.[74, 82]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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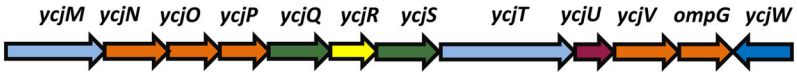


Figure 1.

Organization of the *ycj* gene cluster in *E. coli*. These genes encode proteins that are predicted to include five sugar transporters (YcjN, YcjO, YcjP, YcjV, and OmpG), two NAD$^+$-dependent dehydrogenases (YcjS and YcjQ), two polysaccharide hydrolases/phosphorylases (YcjM and YcjT), an epimerase/isomerase (YcjR), a β-phosphoglucomutase (YcjU), and a LacI-type repressor (YcjW).

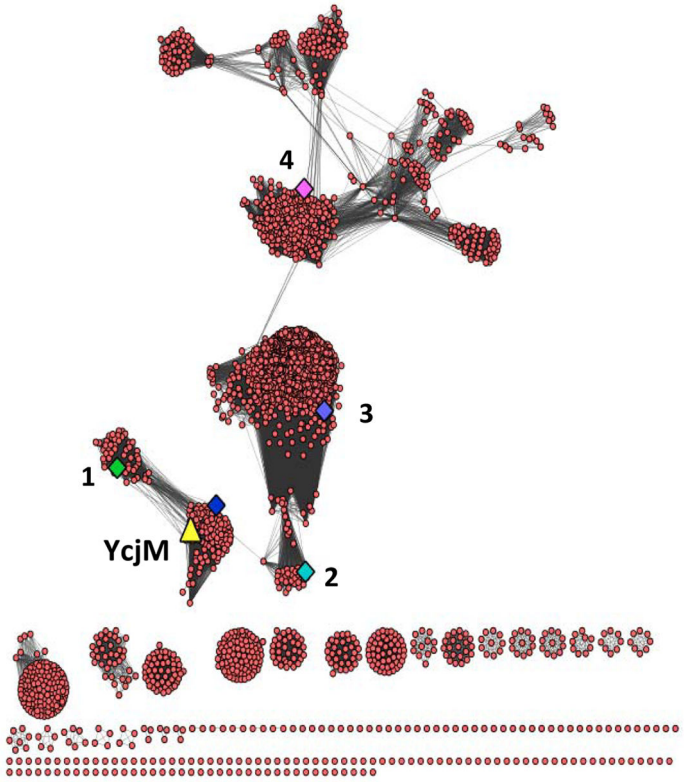


Figure 2.

Sequence similarity network of cog0366 at an E-value cutoff of 1 x 10^{-50}. The network was created using Cytoscape (www.cytoscape.org). Each node represents a non-redundant protein sequence and each edge (or connecting line) represents a BLAST *E*-value between two sequences that is better than the arbitrary value of 1 x 10^{-50}. The lengths of the edges are not significant; in tight clusters the sequences are more closely related as compared to the clusters that contain fewer connections. YcjM is shown as a yellow triangle. Within the same cluster α-(1,2)-glucose-D-glycerate phosphorylase from *Meiothermus silvanus* is indicated with a diamond (dark blue). Diamonds in Groups 1, 2, 3, and 4 contain enzymes that have been experimentally verified. Group 1: sucrose phosphorylase from *Bifidobacterium adolescentis* (green); Group 2: amylosucrase from *Neisseria polysaccharea* (cyan); Group 3: oligo-1,6-glucosidase from *Bacillus subtilis* (light blue); and Group 4: neopullulanase from *Geobacillus stearothermophillus* (pink).

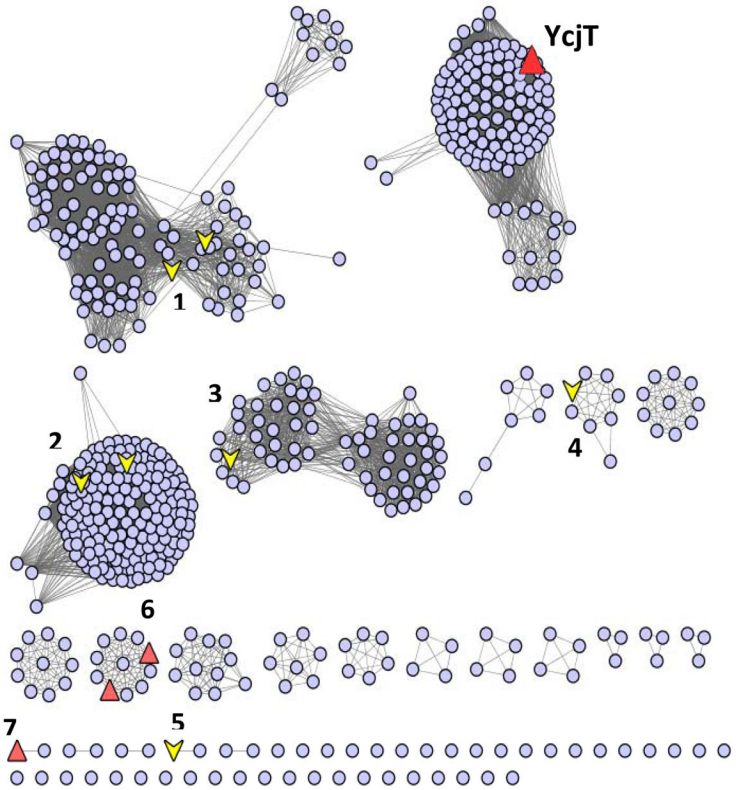


Figure 3.

Sequence similarity network of cog1554 at an E-value cutoff of 1 x 10^{-150}. The network was generated using Cytoscape (www.cytoscape.org). Groups 1, 2, 3, 4, 5, 6, and 7 contain enzymes whose functions are experimentally verified (yellow arrowheads or red triangles). Group 1: α,α-trehalose phosphorylase (*Bacillus stearothermophillus*); Group 2: maltose phosphorylase (*Lactobacillus brevis*); Group 3: trehalose-6-phosphate phosphorylase (*Lactococcus lactis*); Group 4: nigerose phosphorylase (*Lachnoclostridium phytofermantans*); Group 5: α-(1,2)-D-glucose-glycerol phosphorylase (*Bacillus selenitireducens*); Group 6: kojibiose phosphorylase from *Thermoanaerobacter brockii* and *Caldicellulosiruptor saccharolyticus*; Group 7: kojibiose phosphorylase from *Pyrococcus sp*.

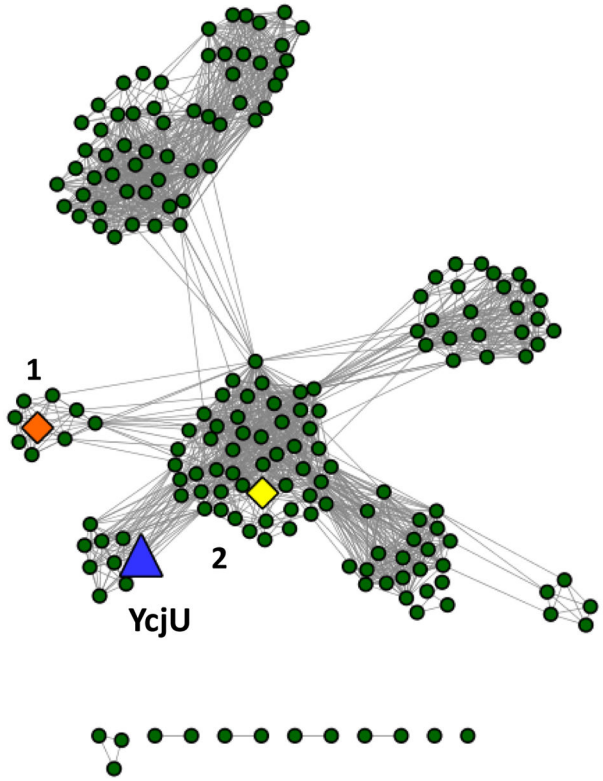
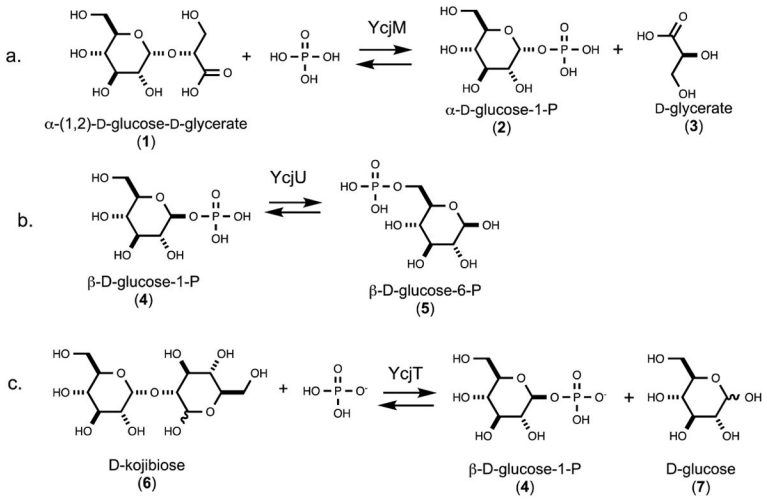
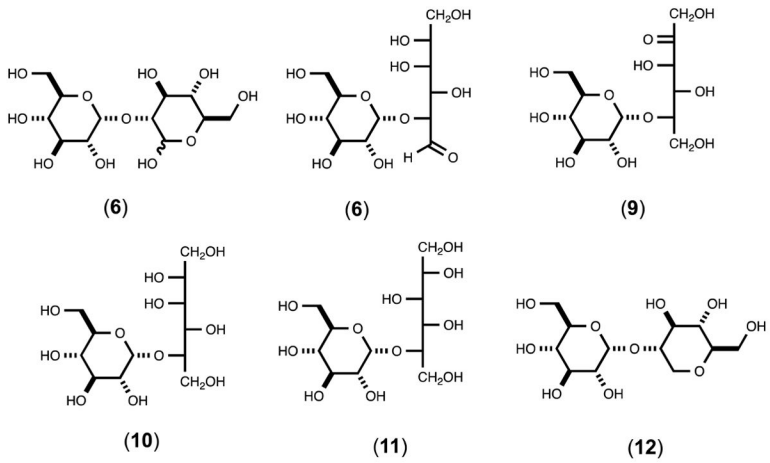


Figure 4.

Sequence similarity network of Interpro family IPR010972 at an *E*-value cutoff of 1 x 10^{-85}. The network was created using Cytoscape (www.cytoscape.org). Diamonds (orange and yellow) represent two experimentally verified β-D-phosphoglucomutase enzymes: PgmB from *Lactococcus lactis* and YvdM from *Bacillus subtilis*. The blue triangle represents YcjU from *Escherichia coli* K-12 MG1655.



Scheme 1.



Scheme 2.

Table 1

Apparent kinetic constants for YcjM (pH 6.5), YcjT (pH 7.5), and YcjU (pH 8.0) at 30 °C.

enzyme	varied substrate	fixed substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
YcjM	α-(1,2)-D-glucose-D-glycerate	phosphate (20 mM)	2.1 ± 0.2	0.069 ± 0.002	(3.1 ± 0.1) x 10^4
YcjM	phosphate	α-(1,2)-D-glucose-D-glycerate (1 mM)	2.4 ± 0.4	2.2 ± 0.5	(1.1 ± 0.2) x 10^3
YcjM	α-D-glucose-1-P	D-glycerate (4 mM)	350 ± 2	9.4 ± 1.4	(3.7 ± 0.3) x 10^4
YcjM	D-glycerate	α-D-glucose-1-P (10 mM)	295 ± 4	4.4 ± 0.5	(6.7 ± 0.5) x 10^4
YcjM	α-D-glucose-1-P	H_2O	2.4 ± 0.3	15.2 ± 0.1	(1.6 ± 0.1) x 10^2
YcjM	D-erythronate	α-D-glucose-1-P (10 mM)	68 ± 1	8 ± 2	(8.5 ± 0.5) x 10^3
YcjT	kojibiose	phosphate (5 mM)	1.1 ± 0.1	1.05 ± 0.03	(1.1 ± 0.1) x 10^3
YcjT	phosphate	kojibiose (5 mM)	0.8 ± 0.1	3.0 ± 0.2	(2.7 ± 0.3) x 10^2
YcjT	β-D-glucose-1-P	H_2O	0.014 ± 0.001	2.01 ± 0.31	7.0 ± 0.1
YcjT	D-glucose	β-D-glucose-1-P (8 mM)	0.8 ± 0.1	1.7 ± 0.2	(2.6 ± 0.1) x 10^2
YcjT	L-sorbose	β-D-glucose-1-P (8 mM)	1.2 ± 0.5	7.9 ± 1.0	(1.5 ± 0.7) x 10^2
YcjT	L-iditol	β-D-glucose-1-P (8 mM)	1.3 ± 0.4	14.0 ± 1.0	92 ± 1
YcjT	D-sorbitol	β-D-glucose-1-P (8 mM)	1.2 ± 0.3	12.0 ± 1.0	(1.0 ± 0.1) x 10^2
YcjT	1,5-anhydro-D-glucitol	β-D-glucose-1-P (8 mM)	1.05 ± 0.03	6.1 ± 0.4	(1.7 ± 0.5) x 10^2
YcjU	β-D-glucose-1-P	---	21 ± 1	0.018 ± 0.001	(1.1 ± 0.1) x 10^6
YcjU	D-glucose-6-P	---	0.5 ± 0.1	1.19 ± 0.04	(4.2 ± 0.1) x 10^2