Title and running title

Identification and characterization of UDP-glucose pyrophosphorylase in *Anabaena* sp.
PCC 7120: UDP-glucose pyrophosphorylase in cyanobacteria

Authors

Yusuke Kawano^{1,3}, Midori Sekine¹ and Masaki Ihara^{1,2*}

1 Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Nagano 399-4511,
Japan, **2** JST, PRESTO, 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012, Japan, **3**
Graduate School of Biological Sciences, Nara Institute of Science and Technology,
8916-5 Takayama, Ikoma, Nara 630-0192, Japan

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Correspondent footnote

E-mail: m_ihara@shinshu-u.ac.jp

Telephone: +81-265-77-1518

Fax: +81-265-77-1518

22 ABSTRACT

23 Exopolysaccharides produced by photosynthetic cyanobacteria have received
24 considerable attention in recent years for their potential applications in the production of
25 renewable biofuels. Particularly, cyanobacterial cellulose is one of the most promising
26 products because it is extracellularly secreted as a non-crystalline form, which can be
27 easily harvested from the media and converted into glucose units. In cyanobacteria, the
28 production of UDP-glucose, the cellulose precursor, is a key step in the cellulose
29 synthesis pathway. UDP-glucose is synthesized from UTP and glucose-1-phosphate
30 (Glc-1P) by UDP-glucose pyrophosphorylase (UGPase), but this pathway in
31 cyanobacteria has not been well characterized. Therefore, to elucidate the overall
32 cellulose biosynthesis pathway in cyanobacteria, we studied the putative UGPase
33 All3274 and seven other putative NDP-sugar pyrophosphorylases (NSPases), All4645,
34 Alr2825, Alr4491, Alr0188, Alr3400, Alr2361, and Alr3921 of *Anabaena* sp. PCC 7120.
35 Assays using the purified recombinant proteins revealed that All3274 exhibited UGPase
36 activity, All4645, Alr2825, Alr4491, Alr0188, and Alr3921 exhibited pyrophosphorylase
37 activities on ADP-glucose, CDP-glucose, dTDP-glucose, GDP-mannose, and
38 UDP-*N*-acetylglucosamine, respectively. Further characterization of All3274 revealed
39 that the k_{cat} for UDP-glucose formation was one or two orders lower than those of other
40 known UGPases. The activity and dimerization tendency of All3274 increased at higher
41 enzyme concentrations, implying catalytic activation by dimerization. However, most
42 interestingly, All3274 dimerization was inhibited by UTP and Glc-1P, but not by
43 UDP-glucose. This study presents the first *in vitro* characterization of a cyanobacterial
44 UGPase, and provides insights into biotechnological attempts to utilize the

photosynthetic production of cellulose from cyanobacteria.

INTRODUCTION

UDP-glucose (UDP-Glc, Fig. 1A) is a ubiquitous biomolecule utilized in most organisms. UDP-Glc is an energetically activated derivative of glucose and acts as a donor of glucosyl residues for glucosylation of diverse cellular molecules (e.g., polysaccharides, glycolipids, and glycoproteins)(1-3). In many organisms, biosynthesis of UDP-Glc from glucose-1-phosphate (Glc-1P) and UTP is catalyzed by UDP-Glc pyrophosphorylase (UGPase, also known as UTP-glucose-1-phosphate uridylyltransferase; EC 2.7.7.9) (Fig. 1A). UDP-Glc serves as a precursor for the synthesis of extracellular polysaccharides and/or lipopolysaccharides that are crucial for bacterial adhesion to host cells. Gene disruption and overexpression studies in bacteria have also confirmed that the UGPase is critical for bacterial virulence and infection (4-7). Therefore, UGPase is considered a potential drug target against infectious bacteria.

Biochemical characterization studies of some bacterial UGPases have revealed insights into important catalytic properties such as the requirement of Mg^{2+} (8, 9), regulation of activity through enzyme multimerization (10), and kinetic parameters of the ordered sequential Bi Bi mechanism (11). However, the physiological biomolecules and metabolites, which act as catalytic effectors of UGPases have yet to be discovered, although they are well known for ADP-glucose pyrophosphorylases (AGPases) (12).

Here, we focused on cyanobacterial UGPase. Cyanobacteria are known for their oxygenic photosynthetic abilities and inhabit various ecological niches such as extreme

environments. In order to survive the physical, chemical, and biological stresses, many cyanobacteria secrete diverse extracellular polysaccharides and form biofilms (13, 14). It is likely that cyanobacterial UGPases mediate these physiological adaptations and responses through polysaccharide production.

Photosynthetic cyanobacteria may be beneficial as a great source of extracellular polysaccharides; therefore, testing the above hypothesis may reveal additional applications in the field of biofuel and renewable energy production. For example, cellulose, a polysaccharide consisting of glucose, is utilized as a source for bioethanol production. At present, cellulose is mainly derived from plants (15), but cyanobacteria are also a potential source of cellulose (16, 17). The probable substrate is UDP-Glc, as is the case in other bacteria (18).

However, not much is known about UGPase in cyanobacteria, or other NDP-sugar pyrophosphorylases (NSPase), with the exception of AGPase (19). Therefore, we sought to study the basic biochemical characteristics of UGPase and NSPase from the filamentous cyanobacteria *Anabaena* sp. PCC 7120 (A. 7120), which can assimilate N₂ from air. In addition, we analyzed the regulation and sequential properties of the purified recombinant putative UGPase, All3274-His.

MATERIALS AND METHODS

Strains and cultures

Escherichia coli BL21 (DE3) (Life Technologies, Carlsbad, CA) was used as the parental strain for recombinant protein expression. Transformed cells were cultured with shaking (120 rpm) in Luria-Bertani (LB) medium, supplemented with 100 µg mL⁻¹

ampicillin (pET22b) or 20 $\mu\text{g mL}^{-1}$ kanamycin (pET28a).

Cloning and construction of plasmids

Coding regions (without the stop codon) of the genes were amplified from the genomic DNA of *Anabaena* sp. PCC 7120 by PCR, using primers containing the corresponding restriction sites (Table S1). The resultant PCR products were digested with *NdeI* and *NotI*, and ligated into pET22b (Merck, Rahway, NJ), digested with the same enzymes. The *alr2825* PCR fragment was cloned directly by In-Fusion cloning (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. In *all4645* and *alr0188*, the original start codon (GTG) was modified to ATG. For construction of His-All4645/pET28a, the C-terminal His tag sequence in All4645-His/pET22b was substituted with a stop codon by inverse PCR and self-ligation. The *all4645* fragment was digested from the resultant plasmid by using *NdeI* and *NotI* and was ligated into pET28a (Merck) cut at the identical sites. All the plasmids were sequenced, and we confirmed that no mutations were introduced, except for a synonymous PCR error in *alr2361* (A1911G). The primers used are listed in Table S1.

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Expression of recombinant enzymes

The *E. coli* BL21 (DE3) strain transformed with plasmids was cultured overnight at 37 °C and used as starter inoculum. For protein expression, the initial inoculum was diluted in LB to achieve an OD₆₀₀ of approximately 0.2, and the main culture was then started at 37 °C. After 1 h (OD₆₀₀ ~0.7), recombinant gene expression was induced by adding 0.01 mM IPTG, the culture temperature was adjusted to 18 °C,

and the culture was continued for a total of 24 h. The cells were harvested by centrifugation, washed with water, and stored at -20 °C.

Purification and preparation of recombinant enzymes

The cells stored at -20 °C were suspended in disruption buffer (50 mM Tris-HCl [pH 7.6], 500 mM NaCl, 20 mM imidazole, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) and disrupted on ice with 5-s sonication cycles (20 W, total: 2 min/g wet cells) with 3-s intervals. The suspension was centrifuged at 22,300 × g at 4 °C for 10 min, and the supernatant was filtered through a 0.45-µm pore size filter. Using AKTA prime plus (GE Healthcare, Little Chalfont, United Kingdom), the filtrate was loaded onto a HisTrap column (GE Healthcare), which was previously equilibrated with the binding buffer (50 mM Tris-HCl [pH 7.6], 500 mM NaCl, 20 mM imidazole). After washing the nonspecifically bound proteins with the wash buffer (50 mM Tris-HCl [pH 7.6], 500 mM NaCl, 50 mM imidazole), the bound recombinant protein was eluted by using an imidazole gradient (linear gradient to 500 mM in 30 min). Fractions containing the recombinant protein were confirmed by SDS-PAGE, collected, and the buffer was exchanged with enzyme buffer (50 mM HEPES -NaOH [pH 7.6], 100 mM NaCl, 10% [v/v] glycerol) to >99.9% by ultrafiltration (Amicon Ultra-15 Centrifugal Filter Units, 10000 NMWL; Merck), and stored at -80 °C. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

SDS-PAGE and western blot analyses

We used gradient gels (5–20%, SuperSepTM Ace; Wako, Osaka, Japan) and the Rapid Stain CBB Kit (Nacalai Tesque, Kyoto, Japan) for SDS-PAGE according to the manufacturer's instructions. For western blot analysis, proteins resolved in the SDS-PAGE gel were transferred onto polyvinylidene difluoride membranes (Immobilon, Merck) for 1 h under 80 mA constant-current conditions. Blots were blocked with 2% (w/v) skim milk in TBST buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% [v/v] Tween 20) and then incubated with a mouse biotin-conjugated anti-histidine-tag antibody (Penta-His Biotin Conjugate; Qiagen, Venlo, Netherlands), followed by incubation with a streptavidin-conjugated HRP secondary antibody (PROzyme, Hayward, California) diluted in the blocking solution. Blots were washed with TBST buffer. Immunodetection of HRP was carried out using Pierce Western Blotting Substrate (Thermo Scientific, Waltham, Massachusetts). The resulting chemiluminescence was detected using the ImageQuant LAS 4000 mini (GE Healthcare).

NDP-sugar pyrophosphorylase assay

The method previously described by Yang et al (20) was used for the NDP-sugar pyrophosphorylase assay. The NDP-sugar formation reaction (forward) mixture consisted of the indicated concentrations of substrates (NTP and sugar 1-phosphate), 5 mM MgCl₂, 100 mM HEPES-NaOH [pH 7.6], 100 mM NaCl, 0.1 U inorganic pyrophosphatase (Sigma-Aldrich, St. Louis, Missouri), and 0.025 μ M purified protein ($\sim 0.85 \mu\text{g mL}^{-1}$ in All3274-His), unless otherwise noted. The NDP-sugar pyrophosphorolysis reaction (reverse), was performed using the same reaction mixture

composition, except that different substrates (UDP-Glc and PPi) were used without pyrophosphatase. The reactions were performed in a total volume of 70 μ L, started with the addition of enzyme into the mixture preincubated at 37 °C, incubated for an additional 20 min or 3 min, and terminated by heating at 100 °C for 2 min. After centrifugation, 20 μ L of the supernatant was analyzed by HPLC. The chemicals used were as follows: (d)NTPs, Jena Bioscience (Jena, Germany); UDP-Glc, Glc-1P, and mannose 1-phosphate, Sigma-Aldrich; and GDP-mannose and UDP-*N*-acetylglucosamine, (Yamasa, Choshi, Japan).

HPLC analysis

HPLC analysis was performed using the Prominence HPLC system (Shimadzu Scientific Instruments, Kyoto, Japan). The reaction products were analyzed by anion-exchange chromatography (TSK-DEAE-5PW, 30- μ m particle; TOSOH, Tokyo, Japan) in a 7.5 mm (internal diameter) $\times$ 75 mm (length) column. The assayed samples were resolved with a linear gradient program of 0.2–500 mM ammonium formate aqueous solutions in mobile phase. (d)NTP and NDP-sugar were detected by absorption at 262 nm derived from their base moiety. The peak assignment was carried out in comparison with the retention times of standard compounds, except for those of CDP-glucose and dTDP-glucose, which were not available. For CDP-glucose and dTDP-glucose, the peaks observed at the similar retention time as other NDP-sugars were considered as the corresponding peaks. The results were quantitated by the peak area measurements by using standard curves.

Determination of kinetic parameters and specific activity

The initial rates (reactions for 2 or 3 min) obtained in the assays were plotted as a function of the substrate concentration. Curve fitting to Michaelis–Menten equation by non-linear regression analysis, and calculations of apparent k_{cat} and K_{m} were executed using IGOR software program (WaveMetrics, Lake Oswego, OR). The initial rates were also used to calculate the specific activities (U/mg enzyme). One unit (U) of enzyme activity is defined as the amount of enzyme required to release 1 μmol of product per minute. The obtained specific activities were plotted as a function of the enzyme concentration to assess the activation of the enzyme depending on the enzyme concentration.

Crosslinking experiment

The crosslinking reactions were carried using N-hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) according to the supplier's instructions (Thermo Scientific). Samples of purified All3274-His were incubated at room temperature for 15 min in 5 mM MgCl_2 , 100 mM HEPES-NaOH (pH 7.6), 0.35~10 μM of enzyme, 1.1 mg/mL Sulfo-NHS, and 0.4 mg/mL EDC in a final volume of 100 μL . Wherever specified, different substrates (UTP, Glc-1P, and UDP-Glc at 1.5, 1.0, and 1.0 mM final concentrations, respectively) were included in the reaction solution. The reaction was stopped by the addition of 1.4 μL of 2-mercaptoethanol (20 mM final concentration). The samples were then diluted to the same protein concentration, separated on SDS-PAGE, and stained using Sil-Best Stain Neo for Protein and Nucleic Acid/PAGE (Nacalai Tesque).

206

207 **Sequence analysis and 3D structure prediction**

208 The gene and protein sequences were retrieved using CyanoBase
209 (<http://genome.kazusa.or.jp/cyanobase>), KEGG (<http://www.genome.jp/kegg/>), and
210 MBGD (<http://mbgd.genome.ad.jp/>) databases. ClustalX program was used for
211 sequence alignment and cluster analysis (neighbor-joining method). In cluster analysis
212 and alignments, abbreviated codes of organism names are shown before protein names,
213 following the designation of MBGD and the sequences compared, as summarized in
214 Table S2. SMART tool (<http://smart.embl-heidelberg.de/>) was used for protein domain
215 structure predictions. Three-dimensional structure data of *Corynebacterium glutamicum*
216 were obtained from RCSB PDB (ID: 2PA4). Homology-modeled 3D structure data of
217 All3274 were obtained from SWISS-MODEL (Repository; template, 2PA4) (21).
218 Protein structures were visualized using PyMOL viewer (<http://www.pymol.org/>).

219

220 **RESULTS**

221 **Cloning, recombinant expression, and purification**

222 Five cyanobacterial genes were identified as “UDP-glucose
223 pyrophosphorylase” or “UTP–glucose-1-phosphate uridylyltransferase” by annotation
224 in the genome database (CyanoBase): *all3274*, *Ava_4927*, *Npun_F1146*, *Tery_2112*, and
225 *NIES39_K04100*. We investigated whether All3274 (Gene ID; 1106872) in A. 7120 is
226 indeed a UGPase, and whether other candidate UGPases exist. The gene *all3274* is 882
227 bp long and is predicted to encode a protein of 293 amino acids with a molecular mass
228 of 32.6 kDa. According to the domain prediction program (SMART), an

NTP_transferase domain spans almost the entire region (Fig. 1B).

To express All3274 in *Escherichia coli*, *all3274* was amplified by PCR from the A. 7120 genome and was cloned into pET22b (Fig. 1C). The resultant construct expressed the recombinant protein fused to a histidine tag at its C-terminal end (All3274-His). The *E. coli* BL21 (DE3) strain transformed with the above construct was grown at a low temperature (18 °C) for 24 h, and protein expression was induced by IPTG.

SDS-PAGE results showed that half of All3274-His was expressed as a soluble protein, and the other half was insoluble (data not shown). Using the soluble fraction, the recombinant protein was purified by Ni²⁺ affinity chromatography. The purity of the protein obtained was confirmed by SDS-PAGE (Fig. 1D), and the observed molecular mass was in agreement with the calculated mass (33.9 kDa). On a western blot (Fig. 1E), the corresponding band was detected by an anti-histidine-tag antibody.

Activity and specificity

Some NSPases, including UGPases, are known to exhibit catalytic activity on a broad range of substrates (4, 8, 10, 22, 23), making it difficult to speculate on substrate specificities on the basis of sequence information. In A. 7120, eight genes, including *all3274*, were annotated as the “NTP_transferase (pfam)” family, which includes the NSPase family, by using a motif search in the database (MBGD). To identify UGPase in A. 7120, All3274 and all seven NSPases were cloned and purified using the same protocol as that used for All3274-His. The All4645 recombinant protein, fused to the histidine tag at the N-terminal end, was also constructed using pET28a

(His-All4645) vector. Using these proteins, we performed preliminary activity assays in the presence (+) or absence (–) of NDP-sugar peak production in HPLC (Table 1). Our results indicate that All3274, All4645, Alr2825, Alr4491, Alr0188, and Alr3921 represent UGPase, AGPase, CDP-glucose pyrophosphorylase, dTDP-glucose pyrophosphorylase (dTGPase), GDP-mannose pyrophosphorylase, and UDP-*N*-acetylglucosamine pyrophosphorylase, respectively. Alr3400-His and Alr2361-His did not exhibit any activity under the experimental conditions used. From these results, we concluded that All3274 possesses UGPase activity, and thus, we focused on All3274-His in subsequent studies.

To confirm the specificity of All3274-His, we performed NDP-sugar-formation assays with more diverse substrates: three types of sugar 1-phosphates and eight types of (d)NTPs, up to a total of 24 combinations. The assayed products were resolved with HPLC equipped with anion-exchange resin columns, and were detected on the basis of absorption derived from their base moieties.

We found that All3274-His yielded a product, only in the combined presence Glc-1P and UTP (Fig. 2A, arrowhead), with concomitant consumption of UTP. As the retention time of the product was the same as that of the UDP-Glc standard (data not shown), we concluded that All3274-His is in fact a UGPase. None of the other substrate combinations tested yielded new peaks or consumed the respective (d)NTPs (Fig. 2A–F). These results collectively suggest that the activity of All 3274-His on UTP and Glc-1P is specific. Likewise, the reaction in reverse direction detected the UDP-Glc pyrophosphorolysis activity of All3274-His (data not shown), which is consistent with known bacterial UGPases with similar properties (4, 24).

275

276 **Kinetic parameters of All3274-His**

277 To determine the kinetic parameters of All3274-His, we examined the initial
278 rates on a series of substrate concentrations. All3274-His followed typical
279 Michaelis-Menten kinetics (data not shown), and the kinetic parameters are listed in
280 Table 2. The catalytic activity (k_{cat}) of All3274-His for UDP-Glc formation was
281 ~10-fold lower than that for UDP-Glc pyrophosphorolysis, and the K_m for UTP in
282 UDP-Glc formation was ~8-fold lower than that for UDP-Glc in UDP-Glc
283 pyrophosphorolysis.

284

285 **Possible regulation of All3274-His**

286 To explore regulatory effectors of All3274-His, we tested the effects of some
287 physiological molecules in UDP-Glc-formation assays (Fig. 3A). In the absence of
288 Mg^{2+} , All3274-His exhibited no activity, consistent with previous studies on other
289 UGPases (25). The ratio of ATP and ADP is an indicator of cellular energy status, and in
290 the presence of ATP or ADP, the activity of All3274-His decreased to ~60%. The ratio
291 of oxidized and reduced forms of NAD or NADP forms reflects cellular redox power.
292 Regardless of their redox status, the presence of either NAD or NADP, resulted in a
293 decrease of All3274-His activity to ~80% or ~50%, respectively. The activity of
294 All3274-His was slightly decreased in the presence of 5 mM DTT, and its activity was
295 not affected by 1 mM 2-oxoglutaric acid, 3-phosphoglyceric acid, and 5-aminolevulinic
296 acid (data not shown).

297 Next, we investigated the relationship between enzyme concentration and

activity. UDP-Glc-formation assays were carried out with a series of enzyme concentrations under saturating substrate conditions, and the specific activities (U/mg enzyme) were plotted against enzyme concentrations (Fig. 3B). As the enzyme concentration increased from 0.0125 μ M to 0.6 μ M, the specific activity also increased 4-fold within this range and was constant at higher concentrations. Similar phenomenon was observed in UGPase from *Xanthomonas* (10). The *Xanthomonas* enzyme exists in two multimeric states, as an active dimer and an inactive monomer. Therefore, the enzyme concentration-dependent behavior observed strongly suggests the existence of two (or more) multimeric states that exert distinct activities in All3274-His. It was previously reported that UTP promotes dimerization of *Xanthomonas* UGPase. Although we presumed a similar regulation for All3274-His, we did not observe UTP-mediated activation or, presumably, any such dimerization in our experimental conditions (data not shown).

Crosslinking experiment

To estimate the protein oligomeric state, All3274-His was incubated under different conditions with linker-less crosslinking reagents, Sulfo-NHS and EDC (Fig. 3C). In the absence of substrates, it was evident that the intensity of the band corresponding to All3274 dimer with a predicted molecular mass of 75 kDa increased with increasing enzyme concentrations. The addition of substrates such as UTP and/or Glc-1P for the UDP-Glc formation reaction, led to significantly decreased dimer band intensities, whereas adding UDP-Glc did not exert any significant effect. These results indicate that the tendency of All3274 to dimerize is directly proportional to the enzyme

concentration, **but it is inhibited** in the presence of UTP and Glc-1P.

Sequence analysis of All3274

To understand the cyanobacterial UGPase at the genetic level, cluster analysis was performed using amino acid sequences (Fig. S1). The members used in the phylogenetic characterization are genes encoding the “NTP_transferase” domain in seven cyanobacterial species and known UGPases in other bacteria. As a result, they are mainly divided into eight clusters, whose annotations correspond to the substrate categories. Three cyanobacterial genes (green) containing All3274 formed a cluster (pink) with known UGPases in other bacteria (blue). This result suggests that the UGPase in cyanobacteria has the same origin as those in other bacteria do; however, cyanobacterial UGPase is distantly related to the other bacterial gene groups. Notably, only a few cyanobacteria possess UGPase homologs (3 of 7 species), although almost all cyanobacteria possess at least one homolog in other categories of NSPases. To validate this observation, we searched for all cyanobacterial UGPase homologs in MBGD and KEGG databases. We found UGPase homologs in 15 of the 72 species (data not shown). **A higher number of species carrying UGPase homologs were found within Nostocales (7/12 species, including A. 7120) and Oscillatoriales (5/9) than those within Chroococcales (2/35).**

At the amino acid level, All3274 exhibited low similarity of 30% with the known UGPase from *Corynebacterium* (cgb:CG1004). However, in comparison with amino acid sequences of bacterial UGPases, the residues involved in substrate binding (light blue) and Mg²⁺ binding (orange) (26) are highly conserved in All3274 (Fig. 4).

This high degree of conservation in the functionally important residues is consistent with All3274 being a functional UGPase.

DISCUSSION

All3274 is the first cyanobacterial UGPase characterized in detail *in vitro*. This study highlights several interesting features in comparison to other known bacterial UGPases. First, All3274 is a UGPase with high specificity for UTP and does not utilize dTTP to yield dTDP-glucose (Fig. 2), whereas many bacterial UGPases can react with dTTP, as well as UTP (4, 8, 10, 22, 23). Second, the UDP-Glc formation activity of All3274-His is lower than those of the known enzymes, and K_m for Glc-1P is much higher than those of the known UGPases. Finally, the activity and the dimerization tendency of All3274-His are dependent on its concentration (Fig. 3C), suggesting that it is activated by dimerization. Despite the similarity in regulation of *Xanthomonas* UGPase (10) and All3274, these enzymes differ in two ways. While pre-incubation with UTP promotes the dimerization of *Xanthomonas* enzyme, enhancing its activity, UTP suppresses the dimerization of All3274-His (Fig. 3C). Furthermore, C-terminal helix of *Xanthomonas* enzyme proved critical for its dimerization, but a corresponding helix does not exist in All3274-His (Fig. 4). In the following sections, we discuss these characteristic features of All3274-His.

Substrate specificity

Compared with UTP, dTTP is structurally similar, and differs by (i) the absence of ribose 2'-hydroxyl group and (ii) the presence of pyrimidine 5-methyl group. According to the 3D structure of corynebacterial UDP-Glc-bound UGPase (26), the

ribose 2'-hydroxyl group is anchored by hydrogen bonds to both Gly 21 and Glu 36 (Fig. S2A, and yellow arrowheads in Fig. 4). These residues are also conserved in UGPases known to exhibit dTGPase activity (Fig. 4, arrows). Despite the lack of dTGPase activity (Fig. 2), these corresponding residues are conserved in All3274-His. Therefore, these residues are not involved in dTTP substrate accessibility of these enzymes. In the corynebacterial UGPase structure (Fig. S2A), there are no residues around the C5 of pyrimidine in UDP-Glc; the nearest residues of C5 of pyrimidine in UDP-Glc are Ca of Gly 117 and amide nitrogen of Leu 118 (3.8 Å, pink arrowheads in Fig. 4). This suggests that dTTP is an acceptable substrate. Indeed, these Gly and Leu residues are completely conserved in UGPase known to exhibit dTGPase activity (Fig. 4, arrows). However, in All3274, although the corresponding Gly is conserved, the corresponding Leu is substituted with Tyr. The 3D structure of All3274, derived by homology modeling using the corynebacterial UGPase (Fig. S2B), reveals that the bulky phenol group of this Tyr 117 protrudes into the UDP-Glc-binding pocket and is situated near C5 of the pyrimidine moiety (~ 3.7 Å). Therefore, in All3274, this Tyr might stereochemically hinder the accession of dTTP with 5-methyl group but not the accession of UTP without 5-methyl group. This is a probable mechanism by which All3274-His UGPase displays high specificity for UDP-Glc and does not possess dTGPase activity.

Enzyme activities

Before discussing the physiological significance of low activity of All3274, we must consider the possible artifacts caused by C-terminal fused histidine tag and

relatively high concentration of NaCl in the assay buffer. Because the interaction between each of the C-terminal helices of UGPase was proposed to be one of the driving forces for multimerization (10), the fusion of histidine tag to C-terminus may disturb or inhibit the interaction. In addition, the high concentration of NaCl also tends to inhibit the multimerization. Although these possibilities cannot be excluded completely, the effects would be limiting for the following reasons: (i) The UDP-Glc formation activity and the dimerization tendency of All3274 notably increased at higher enzyme concentrations (Fig. 3C), indicating that the regulation mechanism through dimerization is fully functional; (ii) all kinetic parameters except for the k_{cat} of UDP-Glc formation and K_m for Glc-1P in All3274-His were comparable with those of other bacteria (details are described below), indicating no significant structural perturbations; and (iii) the preliminary data on the K_m for Glc-1P was determined using partially purified enzyme fractions from A. 7120 (~100 μM) (27), similar to our data. Therefore, we inferred that the activity of All3274 for UDP-Glc formation is significantly lower than those of known bacterial enzymes, even in the activated form at higher enzyme concentrations.

Bacterial UGPases are known to exhibit a variety of substrate affinity characteristics such as the K_m . The known UGPases exhibit K_m values of 8.5 μM (28), 11.9 μM (9), 70 μM (29), 210 μM (10), and 10 mM (12) toward UTP. Comparatively, All3274-His exhibited a K_m value of 42.4 μM (Table 2), similar to that of the known UGPases. The known UGPases exhibit K_m values of 10~60 μM (8-10, 12, 28, 29) toward Glc-1P, whereas All3274-His notably exhibited one order higher K_m (742 μM , Table 2). The known UGPase exhibits K_m values of 380 μM (12), 990 μM (10), and

3320 μM (30) toward UDP-Glc, whereas, in our study, All3274-His exhibited a comparable but moderately higher K_m value (3040 μM , Table 2). The known UGPase exhibits K_m values of 240 μM (30), 480 μM (10), and 800 μM (12), toward pyrophosphate (PPi), similar to that observed for All3274-His in our study (376 μM , Table 2). Taken together, among all the parameters studied, All3274-His exhibited a remarkably higher K_m for Glc-1P.

According to the crystal structure of the catalytic pocket of corynebacterial UGPase, Asp 143 is assumed to anchor the C6 hydroxyl group of a glucose moiety of UDP-Glc via hydrogen bonds (Fig. S2A). However, the corresponding residue in All3274 is substituted by His. The homology model of All3274 suggests that the ϵ or δ nitrogen of His 140 orient in the different direction from the glucose moiety (Fig. S2B). However, the other residues, except Tyr117 in the catalytic pocket were found to overlap with those of the corynebacterial UGPase (Fig. S2C). Hence, it can be speculated that the higher K_m for Glc-1P in All3274-His might be attributed to the loss of the anchor to the glucose moiety by the substitution at the site.

The k_{cat} of All3274-His for UDP-Glc formation (0.6 s^{-1} , Table 2) is much lower than those for other UGPases (40 s^{-1} (10), 69 s^{-1} (28), 93 s^{-1} (9), and 148 s^{-1} (12)). In contrast, All3274-His exhibited almost similar k_{cat} values for UDP-Glc pyrophosphorolysis (6.35 s^{-1} , Table 2), compared with other UGPases (0.55 s^{-1} (12) and 5.7 s^{-1} (10). Most interestingly, the dimerization tendency of All3274-His was diminished by the addition of UTP and Glc-1P, but not by UDP-Glc (Fig. 3C). This observation indicates that in the presence of UTP and Glc-1P, All3274-His dissociates into monomers and is inactive or less active, whereas in the presence of UDP-Glc, it

remains a dimer and exhibits higher UDP-Glc pyrophosphorolysis activity. This further explains its low UDP-Glc formation activity.

On the basis of our results, it can be assumed that the rate of UDP-Glc formation is low under low Glc-1P concentrations, but it remains low even under higher Glc-1P concentrations because of the monomerization of All3274. The UDP-Glc formation rate *in vivo* is likely to be maintained at a low level, regardless of the Glc-1P concentration. When A. 7120 cells are incapable of growth due to nutrient starvation for example, they may still be capable of photosynthetic carbon assimilation. In this case, carbon metabolic flow originated from the Calvin cycle mainly goes to glycogen as a stock of carbon and energy. Glucose-6-phosphate, in the glycolytic pathway, is converted to Glc-1P, then to ADP-glucose, and eventually to glycogen. In order to utilize this elevated cellular Glc-1P exclusively for glycogen synthesis, it might be necessary to limit and maintain a low level of All3274-mediated UDP-Glc formation.

In contrast to other bacterial UGPases that exhibit higher UDP-Glc formation activity than the UDP-Glc pyrophosphorolysis function, All3274 has the comparable K_m/k_{cat} values in both reactions (Table 2). This implies that the *in vivo* role of All3274 is to balance the glucose metabolism.

Regulation of activity

Xanthomonas UGPase exists in two multimeric states, as an active dimer and an inactive monomer, and UTP promotes its dimerization. However, the *Xanthomonas* enzyme with the truncated C-terminal helix was monomeric and inactive (truncated region was indicated by red letters in Fig. 4) (10). In contrast, All3274 does not possess

a C-terminal extended sequence corresponding to the C-terminal helix of *Xanthomonas* enzyme. Nevertheless, it displayed concentration-dependent increased activity and dimerization tendency (Fig. 3B). From these observations, we hypothesized that the multimerization mechanisms might be different between the two enzymes. In fact, UTP inhibits the dimerization of All3274-His. The inhibition of oligomerization by substrates has also been observed in plant UGPases (31-34). However, plants UGPases are active in the monomeric form and inactive in the oligomeric form, which is opposite to All3274. Plant UGPases are activated by their substrates, whereas All3274 is inactivated by its own substrates, thereby suggesting a remarkably unique regulatory mechanism.

We further studied the effectors that can regulate the activity of All3274-His. We found that the presence of ATP and ADP decreased the UGPase activity to ~60% (Fig. 3A). However, this inhibition is relatively smaller than that by other allosteric NSPases, e.g., the activity of AGPase in *Streptomyces* is inhibited to less than 30% by NADPH, and it is activated more than 33-fold by Glc-6P (12). Moreover, in All3274-His, no difference between ATP and ADP, whose ratio reflects cellular energy status, was observed. Similarly, no difference between the oxidized and reduced forms of NAD or NADP (Fig. 3A) and almost no effect on DTT were observed. Therefore, the redox and cellular energy status do not appear to be important factors in enzyme activity. The drops in the activity by the effectors were small, but significant. These may be due to the effect to facilitate the monomerization by the effectors, as well as UTP.

Although the possibility that an unidentified factor functions as an activator (or dimerization factor) cannot be eliminated, we assume that in A. 7120, cellular UGPase activity is mainly regulated at the expression level (All3274 concentration),

and cellular UDP-Glc levels can be controlled by induction of UGPase as reported in other species (35). To confirm this assumption, we are now developing the strains overexpressing All3274 in A. 7120.

Conclusion

We identified and characterized a cyanobacterial UGPase, All3274, for the first time and showed that it exhibits a high specificity for UDP-Glc, but a restricted UDP-Glc formation activity. Even if the low activity might be due to the lack of the unidentified activators in this *in vitro* study, the introduction of highly active UGPase from other species is necessary to create A. 7120 strains with high cellulose productivity. To this end, we are constructing All3274 overexpressing strains of A. 7120 to assess the physiological role, and planning to create recombinant A. 7120 strains, in which the All3274 is replaced by highly active enzyme, for abundant polysaccharide-production.

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References

1. Lai-Kee-Him, J., Chanzy, H., Muller, M., Putaux, J. L., Imai, T., and Bulone, V.: In vitro versus in vivo cellulose microfibrils from plant primary wall synthases:

507 structural differences. *J. Biol. Chem.*, **277**, 36931-36939 (2002).

508 2. **Awai, K., Kakimoto, T., Awai, C., Kaneko, T., Nakamura, Y., Takamiya, K.,**
 509 **Wada, H., and Ohta, H.:** Comparative genomic analysis revealed a gene for
 510 monoglucosyldiacylglycerol synthase, an enzyme for photosynthetic membrane lipid
 511 synthesis in cyanobacteria. *Plant Physiol.*, **141**, 1120-1127 (2006).

512 3. **D'alessio, C., Caramelo, J. J., and Parodi, A. J.:** UDP-Glc:glycoprotein
 513 glucosyltransferase-glucosidase II, the ying-yang of the ER quality control. *Semin. Cell*
 514 *Dev. Biol.*, **21**, 491-499 (2010).

515 4. **Jiang, S. S., Lin, T. Y., Wang, W. B., Liu, M. C., Hsueh, P. R., and Liaw, S. J.:**
 516 Characterization of UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase
 517 mutants of *Proteus mirabilis*: defectiveness in polymyxin B resistance, swarming, and
 518 virulence. *Antimicrob. Agents. Chemother.*, **54**, 2000-2009 (2010).

519 5. **Jayakar, H. R., Parvathareddy, J., Fitzpatrick, E. A., Bina, X. R., Bina, J. E., Re,**
 520 **F., Emery, F. D., and Miller, M. A.:** A galU mutant of *Francisella tularensis* is
 521 attenuated for virulence in a murine pulmonary model of tularemia. *BMC Microbiol.*, **11**,
 522 179 (2011).

523 6. **Levander, F., Svensson, M., and Radstrom, P.:** Enhanced exopolysaccharide
 524 production by metabolic engineering of *Streptococcus thermophilus*. *Appl. Environ.*
 525 *Microbiol.*, **68**, 784-790 (2002).

526 7. **Vilches, S., Canals, R., Wilhelms, M., Salo, M. T., Knirel, Y. A., Vinogradov, E.,**
 527 **Merino, S., and Tomas, J. M.:** Mesophilic *Aeromonas* UDP-glucose
 528 pyrophosphorylase (GalU) mutants show two types of lipopolysaccharide structures and
 529 reduced virulence. *Microbiology*, **153**, 2393-2404 (2007).

530 8. **Mizanur, R. M., and Pohl, N. L.:** A thermostable promiscuous glucose-1-phosphate
 531 uridylyltransferase from *Helicobacter pylori* for the synthesis of nucleotide sugars. *J. Mol.*
 532 *Cat. B-Enzym.*, **50**, 13-19 (2008).

533 9. **Lai, X., Wu, J., Chen, S., Zhang, X., and Wang, H.:** Expression, purification, and
 534 characterization of a functionally active *Mycobacterium tuberculosis* UDP-glucose
 535 pyrophosphorylase. *Protein Expr. Purif.*, **61**, 50-56 (2008).

- 536 10. **Bosco, M. B., Machtey, M., Iglesias, A. A., and Aleanzi, M.:** UDPglucose
537 pyrophosphorylase from *Xanthomonas* spp. Characterization of the enzyme kinetics,
538 structure and inactivation related to oligomeric dissociation. *Biochimie*, **91**, 204-213
539 (2009).
- 540 11. **Kim, H., Choi, J., Kim, T., Lokanath, N. K., Ha, S. C., Suh, S. W., Hwang, H. Y.,**
541 **and Kim, K. K.:** Structural basis for the reaction mechanism of UDP-glucose
542 pyrophosphorylase. *Mol. Cells*, **29**, 397-405 (2010).
- 543 12. **Asencion Diez, M. D., Peiru, S., Demonte, A. M., Gramajo, H., and Iglesias, A.**
544 **A.:** Characterization of recombinant UDP- and ADP-glucose pyrophosphorylases and
545 glycogen synthase to elucidate glucose-1-phosphate partitioning into oligo- and
546 polysaccharides in *Streptomyces coelicolor*. *J. Bacteriol.*, **194**, 1485-1493 (2012).
- 547 13. **Rossi, F., Micheletti, E., Bruno, L., Adhikary, S. P., Albertano, P., and Philipps,**
548 **R. D.:** Characteristics and role of the exocellular polysaccharides produced by five
549 cyanobacteria isolated from phototrophic biofilms growing on stone monuments.
550 *Biofouling*, **28**, 215-224 (2012).
- 551 14. **Ozturk, S., and Aslim, B.:** Modification of exopolysaccharide composition and
552 production by three cyanobacterial isolates under salt stress. *Environ. Sci. Pollut. Res.*
553 *Int.*, **17**, 595-602 (2010).
- 554 15. **Jordan, D. B., Bowman, M. J., Braker, J. D., Dien, B. S., Hector, R. E., Lee, C.**
555 **C., Mertens, J. A., and Wagschal, K.:** Plant cell walls to ethanol. *Biochem. J.*, **442**,
556 241-252 (2012).
- 557 16. **Kawano, Y., Saotome, T., Ochiai, Y., Katayama, M., Narikawa, R., and Ikeuchi,**
558 **M.:** Cellulose accumulation and a cellulose synthase gene are responsible for cell
559 aggregation in the cyanobacterium *Thermosynechococcus vulcanus* RKN. *Plant. Cell*
560 *Physiol.*, **52**, 957-966 (2011).
- 561 17. **Nobles, D. R., Romanovicz, D. K., and Brown, R. M., Jr.:** Cellulose in
562 cyanobacteria. Origin of vascular plant cellulose synthase? *Plant Physiol.*, **127**, 529-542
563 (2001).
- 564 18. **Ross, P., Mayer, R., and Benziman, M.:** Cellulose biosynthesis and function in
565 bacteria. *Microbiol. Rev.*, **55**, 35-58 (1991).

- 566 19. **Casati, D. F., Aon, M. A., and Iglesias, A. A.:** Kinetic and structural analysis of the
567 ultrasensitive behaviour of cyanobacterial ADP-glucose pyrophosphorylase. *Biochem.*
568 *J.*, **350 Pt 1**, 139-147 (2000).
- 569 20. **Yang, T., and Bar-Peled, M.:** Identification of a novel UDP-sugar
570 pyrophosphorylase with a broad substrate specificity in *Trypanosoma cruzi*. *Biochem. J.*,
571 **429**, 533-543 (2010).
- 572 21. **Kiefer, F., Arnold, K., Kunzli, M., Bordoli, L., and Schwede, T.:** The
573 SWISS-MODEL Repository and associated resources. *Nucleic Acids Res.*, **37**,
574 D387-392 (2009).
- 575 22. **Thoden, J. B., and Holden, H. M.:** The molecular architecture of
576 glucose-1-phosphate uridylyltransferase. *Protein Sci.*, **16**, 432-440 (2007).
- 577 23. **Aragao, D., Fialho, A. M., Marques, A. R., Mitchell, E. P., Sa-Correia, I., and**
578 **Fraza, C.:** The complex of *Sphingomonas elodea* ATCC 31461 glucose-1-phosphate
579 uridylyltransferase with glucose-1-phosphate reveals a novel quaternary structure,
580 unique among nucleoside diphosphate-sugar pyrophosphorylase members. *J. Bacteriol.*,
581 **189**, 4520-4528 (2007).
- 582 24. **Bonofiglio, L., Garcia, E., and Mollerach, M.:** Biochemical characterization of
583 the pneumococcal glucose 1-phosphate uridylyltransferase (GalU) essential for capsule
584 biosynthesis. *Curr. Microbiol.*, **51**, 217-221 (2005).
- 585 25. **Hossain, S. A., Tanizawa, K., Kazuta, Y., and Fukui, T.:** Overproduction and
586 characterization of recombinant UDP-glucose pyrophosphorylase from *Escherichia coli*
587 K-12. *J. Biochem.*, **115**, 965-972 (1994).
- 588 26. **Thoden, J. B., and Holden, H. M.:** Active site geometry of glucose-1-phosphate
589 uridylyltransferase. *Protein Sci.*, **16**, 1379-1388 (2007).
- 590 27. **Cumino, A. C., Marcozzi, C., Barreiro, R., and Salerno, G. L.:** Carbon cycling in
591 *Anabaena* sp. PCC 7120. Sucrose synthesis in the heterocysts and possible role in
592 nitrogen fixation. *Plant Physiol.*, **143**, 1385-1397 (2007).
- 593 28. **Ma, Z., Fan, H. J., and Lu, C. P.:** Molecular cloning and analysis of the
594 UDP-Glucose Pyrophosphorylase in *Streptococcus equi* subsp. *zooepidemicus*. *Mol.*

595 Biol. Rep., **38**, 2751-2760 (2011).

596 29. **Weissborn, A. C., Liu, Q., Rumley, M. K., and Kennedy, E. P.:** UTP:
 597 alpha-D-glucose-1-phosphate uridylyltransferase of *Escherichia coli*: isolation and DNA
 598 sequence of the *galU* gene and purification of the enzyme. *J. Bacteriol.*, **176**, 2611-2618
 599 (1994).

600 30. **Koo, H. M., Yim, S. W., Lee, C. S., Pyun, Y. R., and Kim, Y. S.:** Cloning,
 601 sequencing, and expression of UDP-glucose pyrophosphorylase gene from *Acetobacter*
 602 *xylinum* BRC5. *Biosci. Biotechnol. Biochem.*, **64**, 523-529 (2000).

603 31. **Kleczkowski, L. A., Francoise, M., and Wilczynska, M.:** Factors affecting
 604 oligomerization status of UDP-glucose pyrophosphorylase. *Phytochemistry*, **66**,
 605 2815-2821 (2005).

606 32. **McCoy, J. G., Bitto, E., Bingman, C. A., Wesenberg, G. E., Bannen, R. M.,**
 607 **Kondrashov, D. A., and Phillips, G. N. J.:** Structure and dynamics of UDP-glucose
 608 pyrophosphorylase from *Arabidopsis thaliana* with bound UDP-glucose and UTP. *J.*
 609 *Mol. Biol.*, **366**, 830-841 (2007).

610 33. **Meng, M., Fitzek, E., Gajowniczek, A., Wilczynska, M., and Kleczkowski, L.**
 611 **A.:** Domain-specific determinants of catalysis/substrate binding and the oligomerization
 612 status of barley UDP-glucose pyrophosphorylase. *Biochim. Biophys. Acta*, **1794**,
 613 1734-1742 (2009).

614 34. **Decker, D., Meng, M., Gornicka, A., Hofer, A., Wilczynska, M., and**
 615 **Kleczkowski, L. A.:** Substrate kinetics and substrate effects on the quaternary structure
 616 of barley UDP-glucose pyrophosphorylase. *Phytochemistry*, **79**, 39-45 (2012).

617 35. **Rodriguez-Diaz, J., and Yebra, M. J.:** Enhanced UDP-glucose and UDP-galactose
 618 by homologous overexpression of UDP-glucose pyrophosphorylase in *Lactobacillus*
 619 *casei*. *J. Biotechnol.*, **154**, 212-215 (2011).

620

621

622

623 **Figure legends**

624 FIG. 1. Expression and purification of All3274-His in *E. coli*. (A) Reaction formula
625 catalyzed by UDP-Glc pyrophosphorylase. (B) Predicted domain architecture of
626 All3274. (C) Schematic of the expression plasmid. (D) SDS-PAGE of All3274-His after
627 purification. (E) Western blot of All3274-His following purification.

628

629 FIG. 2. Substrate specificity of All3274-His. HPLC profiles of assay products with
630 different sugar 1-phosphates and (d) NTPs are shown. Y-axis shows relative absorption
631 of the base moiety at 262 nm. As sugar 1-phosphate species, glucose-1-phosphate (A,
632 B), mannose 1-phosphate (C, D), and *N*-acetylglucosamine 1-phosphate (E, F) were
633 used at 500 μ M. As the other substrates, (d) NTP, ATP, GTP, CTP, and UTP (A, C, E),
634 and dATP, dGTP, dCTP, and dTTP were used (B, D, F) at 500 μ M. The enzyme
635 concentration was 0.1 μ M, and the reaction time was 30 min. Arrowhead in (A)
636 represents UDP-Glc.

637

638 FIG. 3. Possible regulation of the activity of All3274-His. (A) **Specific activities (U/mg**
639 **enzyme)** of UDP-Glc formation in the presence (+) or absence (–) of the indicated
640 molecules. The assay was performed with 1000 μ M UTP, 2000 μ M Glc-1P, and 0.025
641 μ M enzyme. (B) **Specific activities (U/mg enzyme)** of UDP-Glc formation at indicated
642 enzyme concentrations. The assay was performed with 1000 μ M UTP and 2000 μ M
643 Glc-1P; reaction time of 3 min. Error bars indicate standard errors of means ($n = 3$). (C)
644 SDS-PAGE of All3274-His after **treatment with Sulfo-NHS and EDC crosslinkers**. The
645 purified enzyme was incubated as described in **Materials** and Methods in a reaction

646 solution containing 5 mM MgCl_2 in the presence of All3274-His at 10 (lanes 1 and 4–7),
647 1 (lane 2) and 0.35 μM (lanes 3 and 8–11), and with the addition of no substrates (lanes
648 1–3), 1.5 mM UTP (lanes 4 and 8), 1.0 mM Glc-1P (lanes 5 and 9), 1.0 mM UDP-Glc
649 (lanes 6 and 10), and 1.5 mM UTP and 1.0 mM Glc-1P (lanes 7 and 11).

650

651 FIG. 4. Alignment of UGPases. The members are UGPases shown in Fig. S1. Residues
652 shown in colors are UDP-Glc binding (light blue) (26), Mg^{2+} binding (orange) (26), and
653 Leu 118 of corynebacterial UGPase and the corresponding Tyr in A. 7120 (pink).
654 Arrows represent proteins known to exhibit dTGPase activity. Arrowheads represent the
655 following: yellow, residues binding to the ribose 2'-hydroxyl group of the UDP-Glc in
656 corynebacterial UGPase; pink, residues nearest to C5 of the pyrimidine residue of the
657 UDP-Glc in corynebacterial UGPase; red, residue binding to the C6 hydroxyl group of a
658 glucose moiety of the UDP-Glc in corynebacterial UGPase, but substituted in All3274;
659 blue, variable residues with small hydrophobic side chains, binding to the UDP-Glc in
660 corynebacterial UGPase. Red letter region in C-terminal 21 amino acids in
661 xcb:XC_1930 (*Xanthomonas campestris*) is corresponding to the truncated region that
662 includes the helix required for activity and dimerization in closely related *Xanthomonas*
663 *axonopodis* UGPase.

Figure 1

Figure 2

Figure 3

[illegible]

1 TABLE 1. Preliminary activity assays of recombinant NSPases in *A. 7120*.

Protein	Annotation	Substrates used					
		Sugar 1-phosphate	(d)NTP				
			ATP	GTP	CTP	UTP	dTTP
All3274-His	UDP-glucose pyrophosphorylase	Glc-1P	—	—	—	+	—
All4645-His	ADP-glucose pyrophosphorylase	Glc-1P	—	—	—	—	—
His-All4645	ADP-glucose pyrophosphorylase	Glc-1P	+	—	—	—	—
Alr2825-His	CDP-glucose pyrophosphorylase	Glc-1P	—	—	+	—	—
Alr4491-His	dTDP-glucose pyrophosphorylase	Glc-1P	—	—	—	—	+
Alr0188-His	GDP-mannose pyrophosphorylase	Man-1P	—	+	—	—	—
Alr3400-His	GDP-mannose pyrophosphorylase	Man-1P	—	—	—	—	—
Alr2361-His	GDP-mannose pyrophosphorylase	Man-1P	—	—	—	—	—
Alr3921-His	UDP- <i>N</i> -acetylglucosamine pyrophosphorylase	GlcNAc-1P	—	—	—	+	—

2 Indicated “+” or “—” shows the presence or absence of NDP-sugar-formation activities,
3 respectively. The reaction conditions are as follows: indicated substrate combinations at
4 500 μM each; enzyme concentrations at 5 μg mL⁻¹; overnight reaction; absence of
5 pyrophosphatase. Man-1P: mannose 1-phosphate GlcNAc-1P: *N*-acetylglucosamine
6 1-phosphate.

7
8

1 TABLE 2. Kinetic parameters of All3274-His.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)
UDP-glucose formation			
UTP	42.4 ± 2.00	0.600 ± 0.0691	1.42×10^{-2}
Glucose 1-phosphate	742 ± 155		8.09×10^{-4}
UDP-glucose pyrophosphorolysis			
UDP-glucose	3040 ± 662	6.35 ± 0.328	2.09×10^{-3}
Pyrophosphate	376 ± 107		1.51×10^{-2}

2 Assays were performed using one substrate at varying concentrations and the other
3 substrate at a saturating concentration as follows: 1000 μM UTP, 2000 μM Glc-1P, 2000
4 μM UDP-Glc, and 4000 μM PPi. The assays were performed thrice, and the average
5 value and standard errors are shown.

6

7