The Putative Eukaryote-Like O-GlcNAc Transferase of the Cyanobacterium *Synechococcus elongatus* PCC 7942 Hydrolyzes UDP-GlcNAc and Is Involved in Multiple Cellular Processes

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The posttranslational addition of a single O-linked β -N-acetylglucosamine (O-GlcNAc) to serine or threonine residues regulates numerous metazoan cellular processes. The enzyme responsible for this modification, O-GlcNAc transferase (OGT), is conserved among a wide variety of organisms and is critical for the viability of many eukaryotes. Although OGTs with domain structures similar to those of eukaryotic OGTs are predicted for many bacterial species, the cellular roles of these OGTs are unknown. We have identified a putative OGT in the cyanobacterium *Synechococcus elongatus* PCC 7942 that shows active-site homology and similar domain structure to eukaryotic OGTs. An OGT deletion mutant was created and found to exhibit several phenotypes. Without agitation, mutant cells aggregate and settle out of the medium. The mutant cells have higher free inorganic phosphate levels, wider thylakoid lumen, and differential accumulation of electron-dense inclusion bodies. These phenotypes are rescued by reintroduction of the wild-type OGT but are not fully rescued by OGTs with single amino acid substitutions corresponding to mutations that reduce eukaryotic OGT activity. *S. elongatus* OGT purified from *Escherichia coli* hydrolyzed the sugar donor, UDP-GlcNAc, while the mutant OGTs that did not fully rescue the deletion mutant phenotypes had reduced or no activity. These results suggest that bacterial eukaryote-like OGTs, like their eukaryotic counterparts, influence multiple processes.

Although long believed to be solely attributes of eukaryotes, many glycosylation pathways are now evident in prokaryotes. Bacterial proteins can be modified with a variety of N-linked (1) and O-linked glycans (2). Posttranslational modification of serine or threonine residues with single O-linked β -N-acetylglucosamine (GlcNAc) by O-GlcNAc transferases (OGTs) is common in eukaryotes. All eukaryotic OGTs share a domain structure consisting of tetratricopeptide repeats (TPRs) that are involved in protein-protein interactions (3) followed by the glycosyltransferase catalytic region (Fig. 1A). The catalytic region is composed of two highly conserved domains (4), which are linked by a variable-length insertion. OGT homologs occur across prokaryotic phyla (4), and a number of these OGTs are eukaryote-like (Fig. 1B); this is best illustrated by the similarities in the crystal structures of the *Xanthomonas campestris* (5, 6) and human (7, 8) OGTs. In addition to sharing similar domain structures, key amino acids in and around the active site are conserved in the eukaryotic and bacterial enzymes (see Fig. S1 in the supplemental material).

Unlike other eukaryotic glycosylations, O-GlcNAcylation occurs in the nucleus and cytosol and is dynamically added and removed from proteins. O-GlcNAcylation can regulate a cellular process directly or by acting in competition with phosphorylation (9, 10). Notably, and unlike phosphorylation, which employs a plethora of site-specific kinases and phosphatases, the cycling of O-GlcNAcylation relies on two highly conserved enzymes that add and remove the sugar. An O-GlcNAc hydrolase capable of removing O-GlcNAc modifications from proteins has also been identified in bacteria (11), suggesting that the O-GlcNAc modification may be present and dynamic in prokaryotes. Phenotypes of null or mutant eukaryote-like OGTs have not been reported in prokaryotes, and the biological roles of O-GlcNAcylation in bacteria have not been investigated.

Diverse types of eukaryotic proteins, including transcription factors, cytoskeletal proteins, metabolic enzymes, hormone receptors, and chromatin, are O-GlcNAc modified. The number and range of physiological systems affected by O-GlcNAcylation states are continuously growing (12). Although OGTs were identified over 2 decades ago, the regulation of glycan transfer and how this modification regulates cellular processes are not yet fully understood. Perhaps studying the OGT of a less complex organism will facilitate gaining insights into how O-GlcNAcylation regulates cellular processes.

The photosynthetic cyanobacteria *Synechococcus elongatus* PCC 7942 has one predicted O-GlcNAc transferase (SeOGT), with a characteristic eukaryote-like structure. We describe multiple mutant phenotypes of an OGT deletion strain. We also show homology with eukaryotic OGTs by demonstrating that SeOGT with mutations affecting amino acids important in eukaryotic OGTs does not fully rescue mutant phenotypes. Additionally, we dem-

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FIG 1 Domain organization of eukaryote-like OGTs. (A) Structure of metazoan OGTs, showing N-terminal TPRs and two catalytic regions divided by a variable linker region. (B) Structure of putative prokaryotic OGTs: *Synechococcus elongatus* PCC 7942, *Xanthomonas campestris* pv. *campestris*, *Geobacter* sp. M21, *Thiorhodovibrio* sp. 970, *Bradyrhizobium japonicum*, and *Microcystis aeruginosa*. Gray shading indicates homology to the eukaryotic catalytic domain. Asterisks indicate enzymes with noneukaryotic domain structures. The catalytic domain of the *Bradyrhizobium* protein and *M. aeruginosa* SlpA and SlpB show homology to the *Arabidopsis* OGT SEC but have an uncharacteristic TPR distribution. TPRs were predicted using TPRpred (<http://toolkit.tuebingen.mpg.de/tpred>).

onstrate a decrease in hydrolysis of UDP-GlcNAc by the mutant SeOGTs.

MATERIALS AND METHODS

Bacterial growth. Cultures were grown in BG-11 medium with no antibiotics unless specified (13). Cultures were grown at 22°C under continuous fluorescent light (2,500 lx) with aeration. To aerate, an aquarium air pump (Petco) was used to introduce bubbles into the bottom of culture flasks through a Pasteur pipette plugged with sterile cotton.

Generation of OGT deletion and rescue strains. Constructs used to create deletion and rescue strains were made using MultiSite Gateway three-fragment vectors (Invitrogen) according to the supplier's protocols (Table 1 lists all vectors; see also Table S1 in the supplemental material for a list of primer sequences). Briefly, 600 bp of sequence directly upstream and 600 bp directly downstream of the SeOGT gene (SynPCC7942_0051; GenBank accession number [AB56083.1](https://www.ncbi.nlm.nih.gov/nuccore/AB56083.1)) were used to make pDONR SeOGT 5' and pDONR SeOGT 3', respectively, and a kanamycin resistance cassette was cloned into pDONR 221 to create pDONR Km^r. These constructs were used in a MultiSite Gateway reaction with pDEST R4-R3 vector to create pENTR Δ ogt::Km^r. This construct was transformed into *S. elongatus* PCC 7942 according to a previously established protocol (13). Homologous recombination resulted in replacement of the native gene with the antibiotic marker, which was used to select for the Δ ogt::Km deletion strain (denoted as the Δ ogt strain in the figures) (strains are listed in Table 2). The genomic region was sequenced to confirm full deletion of the SeOGT.

The *Synechococcus* genome has two gene-free sites (neutral sites) where insertion of sequences does not cause any confounding downstream effects (13). For rescue experiments, wild-type and mutant SeOGT genes under the control of the native promoter were introduced into neutral site two (NSII) of the Δ ogt strain. The Softberry bacterial promoter prediction software BPROM (Softberry, Inc., Mount Kisco, NY) predicts that the SeOGT promoter is 293 bp upstream of the start codon. Wild-type SeOGT with 697 bp directly upstream of the start codon was

first fused to a streptomycin-spectinomycin (Strep-Spec) cassette from pCDF Duet-1 (EMD-Millipore), in opposing orientation, using splice overlap extension PCR. This fusion was used to create pDONR pOGT:SeOGT-Spec^r. MultiSite Gateway cloning was used with the following vectors: pDONR pOGT:OGT-Spec^r, pDONR NSII 5', pDONR NSII 3', and pDEST R4-R3, and the resulting construct, pENTR pOGT:OGT-Spec^r, was transformed into the Δ ogt strain. The resulting Δ ogt::Km^rNSII::pSeOGT:SeOGT-Spec^r rescue strain (the Δ ogt::OGT strain in the figures) was selected using spectinomycin. Transformed colonies were screened and genotyped to confirm the presence of the expected sequences. After confirmation of a stable genotype, cultures were no longer grown under drug selection.

Mutant SeOGT rescue constructs were created by splice overlap extension PCR using primers introducing mutations that create single amino acid changes (see Table S1 in the supplemental material). The G350D mutant was then fused to a streptomycin-spectinomycin resistance cassette in opposite orientation to the SeOGT gene as described above for the Δ ogt::OGT strain. For other constructs, the Strep-Spec cassette was fused to the downstream recombination region NSII 3' in the pDONR NSII 3' vector, resulting in the pDONR NSII 3'-Spec^r vector. MultiSite Gateway was used to create the constructs used to generate these mutant OGT strains. The Δ ogt::Km^r NSII::pSeOGT:SeOGT G350D, Δ ogt::Km^r NSII::pSeOGT:SeOGT H280A, and Δ ogt::Km^r NSII::pSeOGT:SeOGT K445A mutant strains are denoted in the figures as G350D, H280A, and K445A, respectively.

Culture settling. To determine rates of settling, 1 ml of cultures at similar initial optical densities at 730 nm (OD_{730}) were transferred into 1-ml spectrophotometric cuvettes. The cuvettes were covered with Parafilm and allowed to sit under continuous fluorescent light (2,500 lx) without agitation or aeration. The OD_{730} was measured at 24-h intervals. Culture growth was determined using paired replicate cultures. Each day one of the replicate cultures was resuspended, and the OD_{730} was measured. In addition, growth was also monitored by dilution plating of the resuspended cultures on solid BG-11 medium to determine CFU counts.

TABLE 1 Plasmids used in this study

Plasmid group and name	Source plasmid	Description or function
Gateway Technology plasmids ^a		
pDONR 221		Donor vector for three-way cloning
pDONR P4 P1		Donor vector for three-way cloning
pDONR P2 P3		Donor vector for three-way cloning
pDEST R3 R4		Destination vector for three-way cloning
pDONR 221		Source of Km ^r gene
pCDF_Duet		Source of Sp ^r gene
pRARE-MBP-DEST		Used for protein expression
Plasmids created in this study		
pDONR Km ^r	pDONR221	Kanamycin resistance cassette
pDONR SeOGT 5'	pDONR	Upstream region of SeOGT for deletion
pDONR SeOGT 3'	pDONR	Downstream region of SeOGT for deletion
pENTR $\Delta ogt::Km^r$	pDEST R3 R4	Final construct for SeOGT deletion transformation
pDONR NSII 5'	pDONR P4 P1	Upstream region of NSII for introduction of genes
pDONR NSII 3'	pDONR P2 P3	Downstream region of NSII for introduction of genes
pDONR NSII 3'-Spec ^r	pDONR P2 P3	Downstream region of NSII for introduction of genes, fused to spectinomycin resistance cassette
pDONR pOGT:SeOGT-Spec ^r	pDONR 221	SeOGT and native promoter, fused to spectinomycin resistance cassette
pENTR pOGT:SeOGT-Spec ^r	pDEST R3 R4	Final construct for $\Delta ogt::SeOGT$ rescue strain
pDONR SeOGT G350D	pDONR 221	Mutant constructs for Δogt rescue strain
pENTR SeOGT G350D	pDEST R3 R4	Mutant constructs for Δogt rescue strain
pDONR SeOGT H280A	pDONR 221	Mutant constructs for Δogt rescue strain
pENTR SeOGT H280A	pDEST R3 R4	Mutant constructs for Δogt rescue strain
pDONR SeOGT K445A	pDONR 221	Mutant constructs for Δogt rescue strain
pENTR SeOGT K445A	pDEST R3 R4	Mutant constructs for Δogt rescue strain
pRARE SeOGT	pRARE	Protein expression of SeOGT in <i>E. coli</i>
pRARE SeOGT G350D	pRARE	Protein expression of SeOGT G350D in <i>E. coli</i>
pRARE SeOGT H280A	pRARE	Protein expression of SeOGT H280A in <i>E. coli</i>
pRARE SeOGT K445A	pRARE	Protein expression of SeOGT K445A in <i>E. coli</i>

^a DONR, donor vector; DEST, destination vector.

Cell aggregation. Cultures were grown as described above to exponential phase with aeration. Fifty microliters of cultures was diluted into 500 μ l of BG-11 medium; 50 μ l of this dilution was applied to the well of a hemocytometer with a coverslip and allowed to sit at 22°C for 20 min. Cells were then counted as either single cells or cell clusters, with the exception of cells with end-to-end contact, which could represent newly divided cells.

TEM imaging. For transmission electron microscopy (TEM) imaging, exponential cultures fixed with 3% glutaraldehyde were prepared for TEM with osmium tetroxide staining according to the methods at Cyanosite (<http://www.cyanosite.bio.purdue.edu/protocols/semithin.html>) and sectioned. Cells were visualized using a Philips CM12 transmission electron microscope and digitally photographed. ImageJ software (<http://rsbweb.nih.gov/ij/>) was used to measure the width of the lumen of cells that presented as round, midcell cross sections.

Production of *Saccharomyces cerevisiae* exopolyphosphatase PPX1. The exopolyphosphatase PPX1 from *Saccharomyces cerevisiae* was cloned into the expression vector pET11c (Invitrogen), expressed in *Escherichia coli* cells (Rosetta; Novagen), and purified according to Wurst et al. (14).

TABLE 2 *S. elongatus* strains created in this study

Strain no.	Description	Name
KS011	$\Delta ogt::Km^r$	Δogt strain
KS012	$\Delta ogt::Km^r$ NSII::pSeOGT:SeOGT-Spec ^r	$\Delta ogt::OGT$ strain
KS013	$\Delta ogt::Km^r$ NSII::pSeOGT:SeOGT-Spec ^r G350D	G350D
KS014	$\Delta ogt::Km^r$ NSII::pSeOGT:SeOGT-Spec ^r H280A	H280A
KS015	$\Delta ogt::Km^r$ NSII::pSeOGT:SeOGT-Spec ^r K445A	K445A

Enzymatic activity was confirmed using commercially synthesized polyphosphate (sodium phosphate glass type 45; Aldrich).

Inorganic phosphate measurements. *S. elongatus* cultures were grown to exponential phase, and cells were pelleted by centrifugation for 15 min at $3,700 \times g$ in a JA-20 rotor (Beckman). The cell pellets were frozen at -80°C , thawed, and resuspended in French press buffer consisting of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 M sodium perchlorate, and 1% (wt/vol) SDS (15); cells were disrupted via two passes through a French press. Cellular debris was cleared by centrifugation at 4°C for 15 min at $20,000 \times g$ in a JA-20 rotor (Beckman), and aliquots of the supernatant were frozen at -20°C .

The amounts of free inorganic phosphate and phosphate in polyphosphate bodies were determined using a modified protocol from Werner et al. (16). Fifty microliters of cell lysate was added to 50 μ l of 1 M sulfuric acid and vortexed briefly. The mixture was incubated for 5 min, then neutralized with 50 μ l of 2 M sodium hydroxide and 100 μ l of red Tris solution (1 M Tris-HCl, pH 7.5, 6% neutral red solution [Ricca Chemical Company, Arlington, TX]), vortexed, and centrifuged to remove precipitated proteins. pH was assessed via color test and adjusted to neutral (acidic solutions are pink, basic solutions are yellow, and neutral pH solutions are pinkish-orange). Fifty-microliter aliquots of this neutralized solution were transferred to two separate microcentrifuge tubes; one aliquot was used to determine the amount of free inorganic phosphate, and the other was used to determine the amount of total inorganic phosphate (free plus polyphosphate). Polyphosphate in one sample was hydrolyzed at 37°C following the addition of 142 μ l of reaction buffer (20 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 5 mM ammonium acetate) and 8 μ l of PPX1. A pilot experiment indicated that the hydrolysis was complete after 60 min, so samples were incubated for 60 min. In some cases, additional polyphosphate was added, and the reaction time was extended for

FIG 2 Mutant cells settle and aggregate. (A) Early exponential-phase cultures were transferred to a cuvette and allowed to sit without agitation for 0 or 4 days before being photographed. (B) Early exponential-phase cultures were transferred to a cuvette and allowed to sit without agitation, and the optical density (OD_{730}) was measured daily (bars). To monitor growth rate, cells in duplicate cuvettes were resuspended before the OD_{730} was measured (lines). (C) Cells were placed on a hemocytometer slide for 20 min and photographed at a magnification of $\times 100$. The arrowheads indicate aggregated cells. Scale bar, 2 μ m. (D) The fraction of cells (wild type, $n = 1,934$; $\Delta og t$ cells, $n = 1,964$; $\Delta og t::OGT$ cells, $n = 1,968$) in aggregates of two or more was determined. In panels B and D, the data represent the results from three complete biological replicates, with the error bars indicating the standard error. WT, wild type.

an additional 60 min to confirm that the reaction was not enzyme limited. The aliquot used to measure free inorganic phosphate was treated similarly to the total phosphate sample except that PPX1 was omitted.

To measure phosphate in these samples, 344 μ l of 28 mM ammonium heptamolybdate in 2.1 M sulfuric acid was added to each tube, and tubes were incubated for 20 min at 22°C. To develop color, 256 μ l of 0.76 mM malachite green oxalate in 0.35% polyvinyl alcohol was added to each sample and incubated for 20 min at 22°C. The OD_{595} was then measured. A standard curve using fresh potassium phosphate solutions was created for each experiment. The amount of inorganic phosphate as polyphosphate was calculated by subtracting the amount of free inorganic phosphate from the amount of total phosphate.

Expression and purification of SeOGT. Wild-type and mutant SeOGT were expressed as maltose-binding protein (MBP) fusion proteins using pRARE-MBP-DEST (17) in *E. coli* BL21(AI) cells. Proteins were purified on amylose columns according to the manufacturer's recommendations (NEB) and flash frozen at -80°C in 20% glycerol. Coomassie-stained SDS-PAGE gels indicated that approximately 90% of the protein in each preparation was full-length SeOGT (data not shown).

Enzyme-catalyzed hydrolysis of UDP-GlcNAc. Purified SeOGT-MBP enzyme was tested for the ability to hydrolyze radiolabeled UDP- $[\text{^3H}]$ GlcNAc in an assay modified from support protocol 4 in Zachara et al. (18). A UDP- $[\text{^3H}]$ GlcNAc solution was made by drying 2 μ Ci of 2 mCi/ml UDP- $[\text{^3H}]$ GlcNAc (specific activity, 20 to 45 Ci, 0.74 to 1.66 TBq/mmol; PerkinElmer) under argon and resuspending it in 2 μ l of 25 mM adenosine mono-phosphate and 48 μ l of 50 mM Tris, pH 6.8, 5 mM MnCl_2 , and 0.5 μ M UDP-GlcNAc. Each 50- μ l reaction mixture included 5 μ l of the UDP- $[\text{^3H}]$ GlcNAc solution (0.5 μ Ci per reaction volume), 6 μ g of purified enzyme, 50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol (DTT), and 12.5 mM MgCl_2 . The reaction mixture was incubated at 30°C, and the reaction was stopped with the addition of 500 μ l of H_2O . In all experiments, reactions were run for 2 h because a preliminary time course

experiment indicated that reactions with wild-type enzyme were 80% complete at this time. After termination, the reaction product was immediately loaded onto a Dowex AG1 resin (PO_4 form) column, which retains UDP-GlcNAc. The column was washed with 3 ml of H_2O , and radioactivity in the wash was measured using a Packard Tri-Carb 2900TR liquid scintillation counter.

RESULTS

***S. elongatus* has a eukaryote-like OGT.** A BLAST search using the catalytic region of the *Arabidopsis* OGT SPY identified a putative *S. elongatus* OGT gene. The SeOGT gene (Synpcc7942_0051) is predicted to encode a 633-amino-acid protein (GenBank accession number [ABB56083](#)) with a eukaryote-like domain structure of six predicted N-terminal tetratricopeptide repeats followed by the C-terminal catalytic region (Fig. 1B). Protein alignment using standard parameters of Emboss matcher, version 6.3.1 (<http://mobyle.pasteur.fr>), shows that SeOGT has 52% similarity to the *Arabidopsis* OGT SPY and 49% similarity to human OGT (*Homo sapiens* OGT [HsOGT]). The active-site domain shows 59% similarity to SPY and 49% similarity to HsOGT. The full protein shows 45% similarity to the previously identified bacterial eukaryote-like OGT from *Xanthomonas campestris* pv. *campestris* (5, 6).

Phenotypes of an SeOGT deletion mutant. We created an SeOGT deletion strain ($\Delta og t$ strain) by replacing the coding region with an antibiotic marker cassette. The $\Delta og t$ mutant displayed no obvious growth defect under standard growth conditions. However, without agitation from shaking or aeration, the mutant cells showed a very distinct phenotype of rapidly settling to the bottom of the culture container (Fig. 2A). When the optical

FIG 3 The $\Delta og t$ mutant has a wider thylakoid lumen. (A) TEM images show $\Delta og t$ mutant cells sharing a typical cell structure with concentric thylakoids around a central chamber, but they display a wider thylakoid lumen (arrowheads). Scale bar, 200 nm. (B) The thylakoid lumen widths of the wild type, $\Delta og t$ mutant, and $\Delta og t$ rescued strain with a SeOGT transgene. One-way analysis of variance with standard error of 16 cells per strain reveals that the thylakoid widths are statistically different ($P < 0.05$) from each other.

density (OD_{730}) at a fixed height above the bottom of cuvettes was monitored, mutant cultures settled, but wild-type cultures did not (Fig. 2B). The initial density of cultures affected the time to completion of settling, which varied from 24 h for mid-exponential-phase cultures to 1 week for saturated cultures. Some settling occurred when wild-type cultures were at high cell densities, possibly due to cell death or aggregation, whereas cultures at lower cell densities showed an initial increase in OD_{730} due to culture growth.

To determine if mutant cells were viable postsettling, cultures were agitated, and the OD_{730} was measured (Fig. 2B). Wild-type and mutant cultures had similar densities at all time points, indicating that the mutant cells were viable and had the same growth rates as the wild-type cells. Further supporting this, the numbers of CFU were similar after 7 days of settling (see Table S2 in the supplemental material). The buoyant densities of mutant and wild-type cells were similar when assessed by isopycnic centrifugation on a Percoll gradient (see Fig. S2 in the supplemental material), suggesting that a difference in buoyancy is not the cause of mutant cell settling. To determine if impaired metabolic function was involved in the settling phenotype, cells were treated with sodium azide, a biocide that acts by inhibiting electron transport, depleting the cells of ATP. Treatment accelerated the settling of mutant and wild-type cells, and they settled at similar rates, which were greater than the rate of untreated $\Delta og t$ cells (see Fig. S3). Therefore, metabolism con-

tributes to the ability of both mutant and wild-type cells to remain in suspension.

Upon inspection under standard light microscopy, mutant cells had no obvious morphological phenotypes. However, when cells were observed on hemocytometer slides, a higher fraction of the mutant cells were aggregated (Fig. 2C and D). To assess possible changes in cell wall or glycocalyx that might lead to increased cell aggregation, cells were examined by TEM. The mutant cells exhibited no obvious defects in cell wall structure or thickness (Fig. 3A), but the thylakoid lumen of mutant cells was swollen (Fig. 3A and B). In addition, mutant cells had differential accumulation of electron-dense bodies between the thylakoid membranes (Fig. 4; see also Fig. S4 in the supplemental material), with larger bodies more frequently deposited in mutant cells than in wild-type cells.

Because polyphosphate bodies (PPBs) are electron dense, we determined the amounts of inorganic phosphate bound in PPBs by using the *S. cerevisiae* exopolyphosphatase PPX1 to hydrolyze polyphosphate and measuring the resultant increase in inorganic phosphate (16). For both wild-type and mutant cells, relatively small amounts of phosphate were released from polyphosphate, and there was no statistically significant difference in the amounts released (Fig. 4B). Surprisingly, free cellular phosphate was nearly 3-fold higher in mutant cells (Fig. 4B).

Transgene rescue of the $\Delta og t$ strain with wild-type and mutant SeOGTs. To determine if the observed phenotypes were at-

FIG 4 The $\Delta og t$ mutant has aberrant electron-dense bodies and accumulates inorganic phosphate. (A) $\Delta og t$ mutant cells display an increase in electron-dense bodies (arrowheads) distributed between the exterior of concentric thylakoid membrane pairs. Scale bar, 250 nm. (B) Free inorganic phosphate and inorganic phosphate released from polyphosphate bodies (PPBs) by exopolyphosphatase were quantitated. Although there is no difference in the amounts of inorganic phosphate released from polyphosphate, mutant cultures contained nearly 3-fold more free inorganic phosphate. The data represent the results from three complete biological replicates, with the error bars indicating the standard error.

FIG 5 Catalytic region mutations affect SeOGT activity. (A) Wild-type and mutant SeOGT genes were introduced into the $\Delta og t$ null mutant, and the resulting strains were tested for rescue of the settling phenotype. Wild-type OGT rescues the settling phenotype, H280A and K445A SeOGTs do not, and G350D weakly rescues the phenotype. (B) Catalytic region mutations affect hydrolysis of UDP-GlcNAc. Wild-type and mutant MBP fusion proteins were purified. Enzymatic activity was measured based on the amount (disintegrations per minute) of [H^3]GlcNAc released from UDP-[H^3]GlcNAc. The values shown are after subtraction of the amount of [H^3]GlcNAc produced in a reaction with purified MBP-GFP. Reactions were performed in triplicate and analyzed by one-way analysis of variance. Different numbers of asterisks indicate that the values are statistically different (all P values are <0.05). In both A and B, the error bars indicate the standard errors.

tributable to deleting the SeOGT gene, we reintroduced the wild-type SeOGT coding region under the control of its predicted promoter into the $\Delta og t$ strain via homologous recombination. The resulting strain was fully rescued for the settling (Fig. 5A) and cell aggregation phenotypes (Fig. 2D), while the thylakoid width defect was partially rescued (Fig. 3B).

A previously described G570D mutation in *Arabidopsis*, *spy-12*, results in plants with altered hormone responses (19). The G570 residue is conserved in many phyla (see Fig. S1 in the supplemental material) and corresponds to G631 and G350 in the human and *S. elongatus* enzymes, respectively. A mutant SeOGT carrying a G350D mutation did not rescue the settling phenotype of the $\Delta og t$ strain (Fig. 5A).

Cocrystal structures for the HsOGT and the bacterial *X. campestris* OGT with various UDP-GlcNAc analogs and substrate peptides have recently been determined (6–8, 20). Based on these structures, several catalytically important residues have been proposed. Two histidines, HsOGT H558 and H498, have been predicted to be important in the transfer of GlcNAc to substrates (5–7). Additionally, a lysine residue, HsOGT K842, was shown to interact with the UDP portion of the sugar donor (6). Mutational studies have shown that each of these residues is critical for full enzymatic activity (5, 6, 8).

Although H498 is not conserved, H558 and K842 are conserved and correspond to H280 and K445 in SeOGT. Introduction of mutant SeOGT genes carrying the H280A and K445A mutations into the $\Delta og t$ strain did not rescue the settling phenotype (Fig. 5A), suggesting that, similar to human OGT, these residues also play an important role in SeOGT activity.

SeOGT hydrolyzes UDP-GlcNAc. To determine if SeOGT has enzymatic activity, we took advantage of the ability of some sugar transferases to hydrolyze the bond between UDP and the sugar moiety (7). Wild-type SeOGT protein and each of the three mutant SeOGTs were expressed in *E. coli* as MBP fusions and purified. Wild-type SeOGT enzyme hydrolyzed more UDP-GlcNAc sugar than either the G350D or K445A enzyme (Fig. 5B). The G350D enzyme was more active than the negative controls (green fluorescent protein [GFP]-MBP fusion protein and heat-killed OGT enzyme), suggesting that it has some residual activity, but the activity of the K445A enzyme was not different from that of

the negative controls. The H280A enzyme hydrolyzed slightly less UDP-GlcNAc but was not significantly different from the wild type.

DISCUSSION

In addition to the eukaryote-like OGTs, there are bacterial OGTs which have different domain structures or share little sequence homology with the eukaryotic OGTs (Fig. 1B) (21, 22). The *mrpC* gene of the cyanobacteria *Microcystis aeruginosa*, which encodes a highly abundant extracellular protein that is important in cell-to-cell contact and may be O-GlcNAcylated, lies in a gene cluster with two proposed glycosyltransferases, *slpA* and *slpB* (21). Both glycosyltransferases share sequence homology with eukaryotic OGTs, but only SlpA contains TPR domains (Fig. 1B). However, SlpA's TPRs have a different arrangement than eukaryotic OGTs. MrpC has two cellular variants: a secreted form and a form associated with the cell surface that is reported to be O-GlcNAc modified. While the O-GlcNAc modification is proposed to be important for cell surface retention, the effects of blocking the modification on protein localization have not been determined. Two bacterial flagellins that may be modified with O-GlcNAc have been reported (22, 23). *Listeria monocytogenes* flagellins were found by mass spectrometry to contain several O-linked N-acetylhexosamine (HexNAc) modifications, and immunostaining suggested that the modifications were O-GlcNAc (22). However, the enzyme responsible for this modification remains unknown. Shen et al. (23) reported a bacterial OGT, GmaR, that interacts with a DNA-binding protein to regulate flagellar motility and that can modify the flagellin. However, GmaR has no sequence homology to the catalytic region of eukaryotic OGTs.

While the crystal structure of a eukaryote-like OGT from *Xanthomonas campestris* has been determined (5, 6), the cellular role(s) of this enzyme is unknown. In *S. elongatus*, deletion of the SeOGT does not affect the growth rate but causes several distinct phenotypes, suggesting that the OGT affects multiple processes. Mutant cells settle out of medium and aggregate to a greater extent than wild-type cells (Fig. 2). The two phenotypes may be interrelated in that a defect in motility could lead to aggregation or vice versa. Marine strains of *S. elongatus* have motility despite having no known locomotion organelles such as cilia or flagella (24, 25).

Several membrane-related mechanisms for *S. elongatus* motility have been proposed, including localized expansion and contraction of the cell membrane (26), acoustic streaming (27), mechanochemical filaments near the inner membrane causing surface waves (28), and a helical rotor model (29). In a *Synechococcus* species related to *S. elongatus* PCC 7942 several genes involved in motility (30), including ABC transporters, glycosyltransferases, and the cell surface polypeptide SwmA, which forms a glycosylated S layer (31), have been identified. Although the motility genes *swmA* and *swmB* are not present in *S. elongatus* PCC 7942, cell surface proteins are likely to have a role in motility. Bacterial glycoproteins residing on the cell surface are often important in both motility-related structures such as flagella and cilia, as well as mediating cell-to-cell contact (32), and therefore could be SeOGT substrates.

The Δ ogt cell settling phenotype is not due to a difference in buoyant density as mutant and wild-type cells had identical buoyant densities in a Percoll gradient (see Fig. S2 in the supplemental material). After treatment with sodium azide, which inhibits cytochrome oxidase and ATP synthesis, the wild-type and mutant settling rates were similar (see Fig. S3). Interestingly, settling of Δ ogt cells was increased by sodium azide, indicating that Δ ogt cells are only partially impaired in the ability to remain in suspension.

TEM imaging reveals several macromolecular mutant phenotypes. Mutant cells showed a widening of the thylakoid lumen (Fig. 3A and B) and had a defect in the size and number of electron-dense bodies (Fig. 4A; see also Fig. S4 in the supplemental material), which we hypothesized were polyphosphate bodies. However, we did not detect a change in the overall amount of phosphate in the form of polyphosphate, suggesting that the electron-dense bodies are not polyphosphate bodies. Surprisingly, the amount of free inorganic phosphate was significantly higher in the Δ ogt strain (Fig. 4B), suggesting a defect in phosphate uptake or storage by the mutant. Supporting this hypothesis, when cells were transferred to phosphate-poor medium, the settling rate of Δ ogt cells was unaffected, while that of wild-type cells increased (see Fig. S5 in the supplemental material). Supplementing the medium with additional phosphate did not rescue the Δ ogt cell settling phenotype (data not shown). Since phosphate is a key limiting nutrient in many natural environments, regulation of sensing, uptake, and storage of phosphate in bacteria is tightly controlled by a process that involves phosphorylation of signaling proteins (33). Since O-GlcNAcylation and phosphorylation are reciprocal regulatory modifications in eukaryotes (9, 10), perhaps the interplay of O-GlcNAcylation and phosphorylation regulates phosphate status in bacteria.

Mutational studies as well as crystal structure models of OGTs with reaction components have led to identification of several critical amino acids (see Fig. S1 in the supplemental material). Although reintroduction of the native SeOGT gene rescued the settling phenotype, introduction of two genes with mutations predicted to impair catalytic activity did not fully rescue the phenotype (Fig. 5A). The H280A mutation, which affects a residue in or near the active site that possibly interacts with substrate proteins, prevented rescue. Likewise, the K445A mutation, which affects a residue predicted to stabilize the sugar donor, prevented rescue. In contrast, the G350D mutation affects the active-site lid, and cells expressing the mutant protein were weakly rescued. G350 is conserved in the *Arabidopsis* OGT SPY and is mutated to a D in the *spy-12* mutant. Interestingly, the *spy-12* mutation produces a

weak phenotype, suggesting that it causes only a partial loss of OGT activity (19).

Since HsOGT has been reported to hydrolyze UDP-GlcNAc (7), the ability of SeOGT to hydrolyze UDP-GlcNAc was assessed. SeOGT hydrolyzed UDP-[³H]GlcNAc (Fig. 5B); furthermore, single amino acid mutations in the active-site lid (G350D) and in the catalytic domain (K445A) significantly decreased this activity (Fig. 5B). These results support the homology of SeOGT with eukaryotic OGTs. Although the G350 residue resides in the active-site lid, the reduction in activity may be due to instability in the binding pocket or to inhibition of access of the UDP-GlcNAc to the donor binding site. The reduction in activity for the K445 residue is consistent with its predicted role to stabilize the UDP of the donor sugar moiety in the active site. The loss of hydrolytic ability in these mutant enzymes and the inability of the mutant strains to fully rescue mutant phenotypes suggest that catalytic activity is required for SeOGT function.

Interestingly, an additional active-site mutant, H280A, did not have a significant difference in hydrolytic activity from that of the wild type. In HsOGT this residue corresponds to H558 and is critical for modification of proteins (6–8). The residue was initially proposed to serve as a general base for catalysis (5, 7); this residue was later found to be too distant from the acceptor hydroxyl to serve this role and therefore may not be directly involved in hydrolysis (8), which may explain why the mutant protein remains able to hydrolyze UDP-GlcNAc. The H280 strain did not rescue the null mutant settling phenotype (Fig. 5A), suggesting that although the mutant protein is able to hydrolyze UDP-GlcNAc, it may be unable to transfer the sugar to the substrate protein.

Several methods are currently used to identify O-GlcNAcylated proteins, including transfer of labeled galactose to a known terminal O-GlcNAc, the use of antibodies against the O-GlcNAc modification, and chemi-enzymatic methods (18). The GalT assay uses the enzyme β -1,4-galactosyl transferase, which adds a labeled galactose to proteins with glycosylations terminating in GlcNAc. The BEMAD (mild beta-elimination followed by Michael addition) method involves the elimination of the β -O-linkage between glycosylated serines or threonines and substitution at this site with dithiothreitol or biotin, which labels any O linkage. Using these methods, many *S. elongatus* proteins were detected, but wild-type and mutant samples showed the same labeling patterns (data not shown). Several antibodies are currently available to detect the O-GlcNAc modification (18). Western blotting using these antibodies detected many proteins but did not reveal differences between the mutant and wild type (data not shown). Since *S. elongatus* PCC 7942 has no other predicted OGTs, either the proteins detected are modified by a novel OGT or the antibodies are recognizing other structures, as has been previously reported (34). In view of the failure of these approaches to identify O-GlcNAcylation differences between the wild-type and Δ ogt strains, it is likely that glycoproteomic approaches employing mass spectrometry will be needed to identify the SeOGT substrates. Since prokaryotic substrates are not known, we attempted to modify known eukaryotic OGT substrates using *E. coli*-expressed SeOGT but were unsuccessful (data not shown), suggesting that the enzyme either is not an OGT or that bacterial and eukaryotic OGTs have different substrate specificities.

The availability of a single-celled system for the study of O-

GlcNAcylation could accelerate research into the function of this protein modification. For this purpose, neither yeast nor *E. coli* is a viable model organism as both organisms lack OGT. The *S. elongatus* OGT has demonstrated mutant phenotypes, suggesting multiple cellular roles, and can hydrolyze UDP-GlcNAc. Conserved amino acids important for OGT function in mammals and plants are also important in the SeOGT. Therefore, studies of the *S. elongatus* PCC 7942 OGT will potentially contribute to the understanding of both bacterial and eukaryotic OGTs.

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