

# Engineering Ribonucleoside Triphosphate Specificity in a Thymidyltransferase<sup>†</sup>

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**ABSTRACT:** Nature's glycosylation catalysts, glycosyltransferases, indirectly manipulate and control many important biological processes by transferring sugar nucleotide donors onto acceptors. Challenging chemical synthesis impedes synthetic access to sugar nucleotides and limits the study of many glycosyltransferases. Enzymatic access to sugar nucleotides is a rapidly expanding avenue of research, limited only by the substrate specificity of the enzyme. We have explored the promiscuous thymidyltransferase from *Streptococcus pneumoniae*, Cps2L, and enhanced its uridylyltransferase and guanidyltransferase activities by active site engineering. Mutagenesis at position Q24 resulted in a variant with 10-, 3-, and 2-fold enhancement of UDP-glucosamine, UDP-mannose, and UDP-*N*-acetylglucosamine production, respectively. New catalytic activities were observed for the Cps2L variant over the wild-type enzyme, including the formation of GDP-mannose. The variant was evaluated as a catalyst for the formation of a series of dTDP- and UDP-furanoses and notably produced dTDP-Galf in 90% yield and UDP-Araf in 30% yield after 12 h. A series of 3-*O*-alkylglucose 1-phosphates were also evaluated as substrates, and notable conversions to UDP-3-*O*-methylglucose and UDP-3-*O*-dodecylglucose were achieved with the variant but not the wild-type enzyme. The Q24S variant also enhanced essentially all thymidyltransferase activities relative to the wild-type enzyme. Comparison of active sites of uridylyltransferases and thymidyltransferases with products bound indicate the Q24S variant to be a new approach in broadening nucleotidyltransferase activity.

Carbohydrates are more than energy sources within living systems (1). They play critical roles in mediating cell–cell communication through glycoprotein and glycolipid function (2). In bacteria, the lipopolysaccharide and O-antigen are assembled primarily from carbohydrate building blocks (3, 4). Carbohydrates are critical to the activity of many natural products that have been developed into medicines (5). Understanding the glycosylation processes to furnish these glycosylated molecules has been limited, in part, by the lack of access to the appropriate sugar nucleotide substrates required to probe the biosynthetic machinery. The use of nucleotidyltransferases to yield UDP-sugars will provide important enzyme substrates for probing key biosynthetic processes, including lipopolysaccharide (6), O-antigen (7), or mycobacterial galactan (8) formation. In addition, UDP-sugars are potentially capable of activating specific classes of G-protein-coupled receptors, providing new therapeutic avenues (9).

Enzymatic approaches to the synthesis of sugar nucleotides principally involve nucleotidyltransferases from primary metabolism (10–16). Physiologically, these enzymes couple glucose 1-phosphate with a specific nucleoside triphosphate to furnish the NDP-sugar<sup>1</sup> (Figure 1). This is accomplished through a bi-bi ordered mechanism and the formation of a trigonal bipyrimidal phosphoryl ternary complex where the nucleoside triphosphate binds first and the sugar nucleotide is released last (17, 18). Enzymes in the NTP-transferase superfamily include bacterial thymidyltransferases (e.g., RmlA and Cps2L), uridylyltransferases [e.g., Galf, GalU (19), and GlmU (20)], adenylyltransferases [GlgC (21)], and paralogous thymidyltransferases (RffH). All of these enzymes adopt the same common fold (22), maintain the same key catalytic residues, and require Mg<sup>2+</sup>, which

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<sup>1</sup> Abbreviations: ESI-MS/MS, electrospray ionization tandem mass spectrometry; NTP, nucleoside 5'-triphosphate; NDP, nucleoside 5'-diphosphate; NMP, nucleoside 5'-monophosphate; dTTP, deoxythymidine 5'-triphosphate; UTP, uridine 5'-triphosphate; CTP, cytidine 5'-triphosphate; GTP, guanosine 5'-triphosphate; GDP, guanosine diphosphate; α-D-Glc-1-P, α-D-glucopyranose 1-phosphate; GlcN-1-P, α-D-glucosamine 1-phosphate; GlcNAc-1-P, *N*-acetyl α-D-glucosamine 1-phosphate; α-D-Man-1-P, α-D-mannose 1-phosphate; dTDP-α-D-Glc, deoxythymidine α-D-glucopyranose; α/β-D-Glc-1-P, α/β-D-glucofuranose 1-phosphate; α/β-D-Galf-1-P, α/β-D-galactofuranose 1-phosphate; α/β-L-Araf-1-P, α/β-L-arabinofuranose 1-phosphate; α/β-D-Fuc-1-P, α/β-D-6-deoxygalactofuranose 1-phosphate; α/β-D-6Ffuc-1-P, α/β-D-6-deoxy-6-fluorogalactofuranose 1-phosphate; dTDP-α-D-Galf, deoxythymidine α-D-galactofuranose; dTDP-α-L-Araf, deoxythymidine α-L-arabinofuranose.

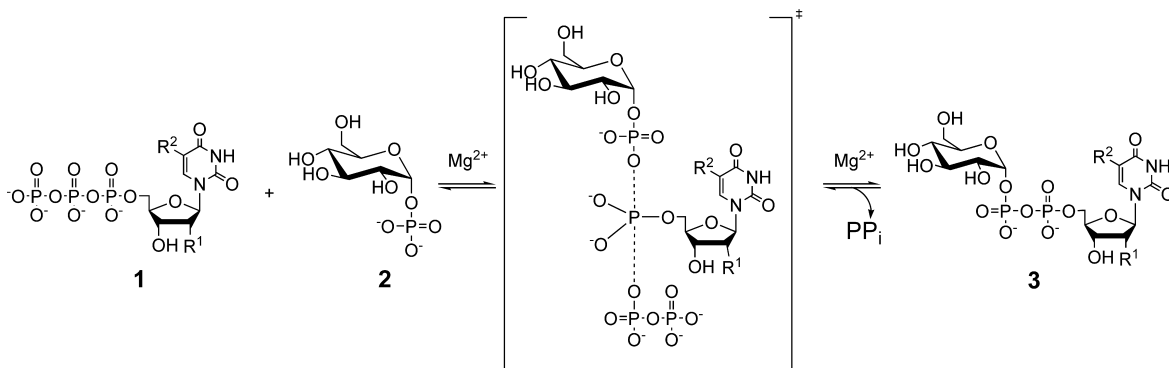


FIGURE 1: Mechanism of nucleotidyltransferases. For thymidyltransferases, dTTP =  $R^1 = \text{H}$  and  $R^2 = \text{CH}_3$ ; for uridyltransferases, UTP =  $R^1 = \text{OH}$  and  $R^2 = \text{H}$ .

suggests a common mechanism (23). The intrinsically broad substrate specificity of the thymidyltransferase (RmlA) enzymes toward a variety of sugar 1-phosphates enables them to forge a selection of sugar nucleotides (24). These studies have primarily focused on the development of dTDP-sugars for natural product glycodiversification and glycorandomization studies (25). Site-directed mutagenesis has further expanded the utility of nucleotidyltransferases toward more structurally diverse sugar 1-phosphates via the engineering of the sugar 1-phosphate binding pocket (26, 27). As a result of the small structural differences between dTTP and UTP, namely, the presence of a C2 hydroxyl functionality on the ribose ring and the lack of the methyl substituent on the pyrimidine base, wild-type thymidyltransferases produce UDP-sugars, providing the sugar 1-phosphates closely resemble glucose 1-phosphate. More synthetically divergent substrates produce significantly lower yields, or a complete lack of conversion (28, 29).

Thorson and Moretti engineered the active site of a thymidyltransferase, RmlA, from *Salmonella enterica* to better accommodate purine bases by site-directed mutagenesis (30). The best of these mutants exhibited a 10-fold improvement in activity for ATP over the wild-type enzyme, and interestingly, one mutant exhibited a 1.3-fold improvement for UTP; however, it remained unclear whether these improvements in nucleoside triphosphate specificity extended to reactions with sugar 1-phosphates other than glucose 1-phosphate. We are unaware of any previous studies that have probed how enzyme mutations improving relative activity observed with nucleoside triphosphate and sugar 1-phosphate substrates will translate into corresponding improvements in activity with a variety of sugar 1-phosphates replacing the glucose 1-phosphate. In this work, we have explored the crystallographic active site of a thymidyltransferase from *Pseudomonas aeruginosa*, RmlA (17), and used this insight to engineer the active site of a homologous thymidyltransferase from *Streptococcus pneumoniae*, Cps2L, to better accommodate ribo-configured nucleoside triphosphates. We evaluated the mutants with a range of structurally diverse substrates to catalyze a range of challenging non-physiological reactions. Our results provide insight into the potential development of a universal nucleotidyltransferase catalyst and contrast the approach taken by Nature in uridyltransferases (31).

## EXPERIMENTAL PROCEDURES

The wild-type His<sub>6</sub>-tagged *cps2L* gene in a pET28a vector (pSK001) (13) was mutated using a QuikChange II Mu-

tagenesis Kit following the manufacturer's instructions (Stratagene). The following primers were used: Q24D, 5'-cttactcgagctacatcgaaaGATctgatgccggtttatg-3' (forward) and 5'-cataaaccggcatcagATCtttcgatgtagctcgagtaag-3' (reverse); Q24S, 5'-cttactcgagctacatcgaaaAGCctgatgccggtttatg-3' (forward) and 5'-cataaaccggcatcagGCTtttcgatgtagctcgagtaag-3' (reverse). *Escherichia coli* DH5 $\alpha$  competent cells were transformed with resulting PCR products and grown overnight on LB medium supplemented with 50  $\mu\text{g}/\text{mL}$  kanamycin. Genetic mutations were confirmed by digestion with PvuII (Q24S) followed by DNA sequencing. Enzymes were harvested as described previously (13). N-Terminally His<sub>6</sub>-tagged proteins were purified by FPLC by elution with increasing concentrations of imidazole in a stepwise gradient (from 25 to 250 mM) at 4  $^{\circ}\text{C}$ . Residual imidazole was removed using a PD10 desalting column (Amersham Biosciences) following the manufacturer's instructions. Reactions were performed as described previously using either 1 or 10 EU (1 EU is the amount of enzyme required to catalyze the conversion of 1  $\mu\text{mol}$  of dTTP and  $\alpha$ -D-Glc-1-P per minute) of purified enzyme in a 50  $\mu\text{L}$  solution containing 1.0 mM NTP, 2.0 mM sugar 1-phosphate, 2.2 mM  $\text{MgCl}_2$ , and 0.5 EU inorganic pyrophosphatase. Reaction progress was monitored by HPLC at 254 nm. For substrates 12–18, a linear gradient from 80/20 to 65/35 A/B over 9.0 min with a subsequent increase to 40/60 A/B from 9.0 to 10.0 min followed by a plateau at 40/60 A/B from 10.0 to 11.0 min at 1.0 mL/min was used. For all other compounds, a linear gradient from 90/10 to 40/60 A/B over 8.0 min followed by a plateau at 40/60 A/B from 8.0 to 10.0 min at 1.0 mL/min was used. Buffer A consisted of 12 mM  $\text{Bu}_4\text{NBr}$ , 10 mM  $\text{KH}_2\text{PO}_4$ , and 5% HPLC-grade  $\text{CH}_3\text{CN}$  (pH 4.0), and buffer B consisted of HPLC-grade  $\text{CH}_3\text{CN}$  (13, 28, 29). Conversions were calculated by dividing the area of the apparent product peak by the total area of the product and substrate peaks combined. ESI-MS/MS was used to confirm the mass and fragmentation of the products, using previously described parameters (28, 29, 32).

## RESULTS

The active site of *S. pneumoniae* Cps2L used in this study and the active site of *P. aeruginosa* RmlA that has previously been described crystallographically have identical amino acids in the nucleoside triphosphate binding site and are 89% identical overall. Analysis of the crystal structure of the *P. aeruginosa* RmlA enzyme bound to dTDP-Glc indicated that the deoxyribose ring was in close contact with residue Q26

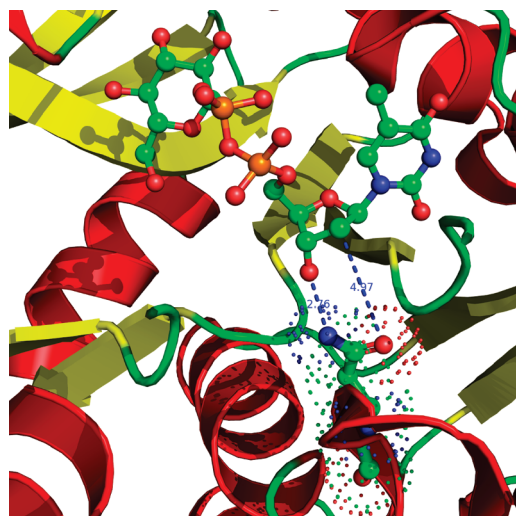


FIGURE 2: Structure of *P. aeruginosa* RmlA [Protein Data Bank (PDB) entry 1g1l] indicating the position of Q26 in the nucleoside triphosphate binding site. dTDP-Glc and Q26 are represented as balls and sticks.

(Figure 2). The primary amide was positioned below the ribose C3-OH group and potentially involved in hydrogen bonding. The same residue in the homologous enzyme, Cps2L, is residue Q24. In an effort to broaden the substrate specificity of the Cps2L thymidyltransferase from 2-deoxyribose toward ribose-containing nucleoside triphosphates, two point mutations were generated: Q24S and Q24D. We reasoned that the Q24S mutant would provide additional space within the active site but still maintain a potential for hydrogen bonding, while the Q24D mutant could facilitate a bifurcated hydrogen bond to the hydroxyl substituents on the ribose ring. We focused our engineering efforts on the ribose, rather than the base functionality, due to the fact that there were few contacts with the pyrimidine methyl substituent in the crystal structure and that removal of the methyl substituent would have a weaker effect. Both mutants were expressed and were purified under standard literature metal affinity chromatography conditions together with fresh wild-type Cps2L (13). Expression levels of Cps2L were as follows: 104 mg/L for the wild type, 128 mg/L for Q24S, and 19 mg/L for Q24D.

The wild-type Cps2L enzyme and the Q24S and Q24D mutants were evaluated as catalysts for the physiological reaction by monitoring the production of dTDP-glucose. These reactions were used to determine the specific activity of each mutant and wild-type enzyme. The Q24S mutant exhibited levels of activity comparable to that of the wild-type enzyme (9 and 13 EU, respectively), while the Q24D mutant was significantly less active (0.4 EU). Both mutants and the wild-type Cps2L enzyme were then evaluated against a panel of commercially available sugar 1-phosphates (4–7) and selected nucleoside triphosphates. After 30 min, significant conversion was obtained with 1 EU of the wild type or Q24S mutant, whereas 24 h was required for similar levels of activity with the Q24D mutant to be reached (Figure 4). Consequently, analysis focused on the Q24S mutant thereafter. The Q24S mutant exhibited almost universally improved relative activity over the wild-type enzyme for the formation of dTDP-sugars. Essentially quantitative conversions were observed with Glc-1-P, GlcN-1-P, GlcNAc-1-P, Gal-1-P, and Man-1-P (2 and 4–7) with dTTP for the Q24S

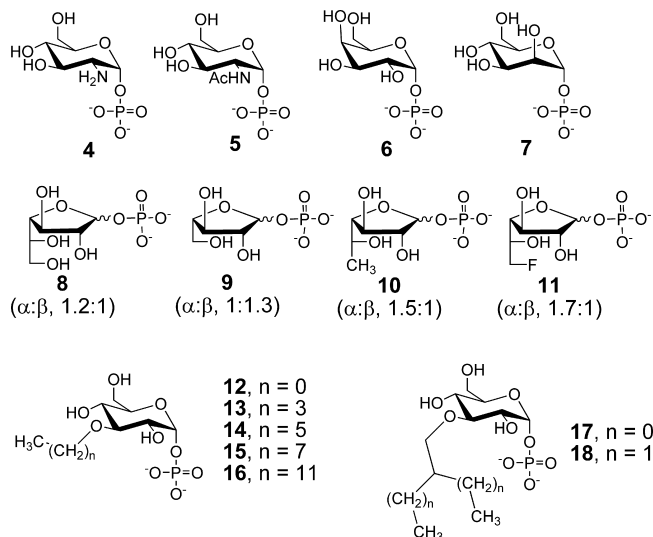


FIGURE 3: Structures of sugar 1-phosphates used in this study. The furanosyl phosphates (8–11) were mixtures of anomers.

mutant-catalyzed reactions, while the wild-type enzyme quantitatively converted only Glc-1-P and GlcN-1-P and had more modest conversions for the remaining substrates. While our Q24S mutation focused upon the nucleoside triphosphate binding site, it substantially enhanced the relative activity of the enzyme toward these sugar 1-phosphates. The effects on sugar nucleotide production by Q24S and the wild-type enzyme with the same commercial sugar 1-phosphates and UTP indicated both enzymes had comparable activity using Glc-1-P and UTP; however, Q24S demonstrated a 10% conversion increase over the wild-type enzyme with UTP and GlcN-1-P. More significantly, Q24S demonstrated >55% conversion with UTP and Man-1-P and >35% with UTP and GlcNAc-1-P. The wild-type enzyme was unable to catalyze either of these two reactions. These results indicate that the Q24S mutation had a significantly beneficial effect on the ability to convert nonphysiological sugar 1-phosphate substrates with nonphysiological nucleoside triphosphates. Of the remaining nucleoside triphosphates evaluated (ATP, GTP, and CTP), only low levels of conversion (<10%) were observed over 30 min, although the mutant outperformed the wild-type enzyme consistently. The exception to the low conversions was the production of GDP-Man, where >40% was produced in 30 min by the mutant and no activity was observed with the wild-type enzyme. These results clearly indicate that the Q24S mutation enhances the ability of the enzyme to catalyze sugar nucleotide production with deoxyribo-configured nucleoside triphosphates, in addition to enhancing production of sugar nucleotides from ribo-configured nucleoside triphosphates, as anticipated from our analysis of the crystallographic data.

The Q24S mutant was also evaluated as a catalyst against two panels of noncommercially available sugar 1-phosphates: a series of furanosyl 1-phosphates (8–11) and a series of 3-O-alkylglucose 1-phosphates (12–18) (Figure 5). The wild-type enzyme was capable of recognizing these two panels of sugar 1-phosphates and produced the corresponding dTDP-sugars; however, in previous studies, conversions with UTP essentially failed with the furanosyl 1-phosphates and were severely limited (<15%) with the 3-O-alkylglucose 1-phosphates. The Q24S mutant was evaluated first with the furanosyl 1-phosphates and dTTP. The order of furanosyl

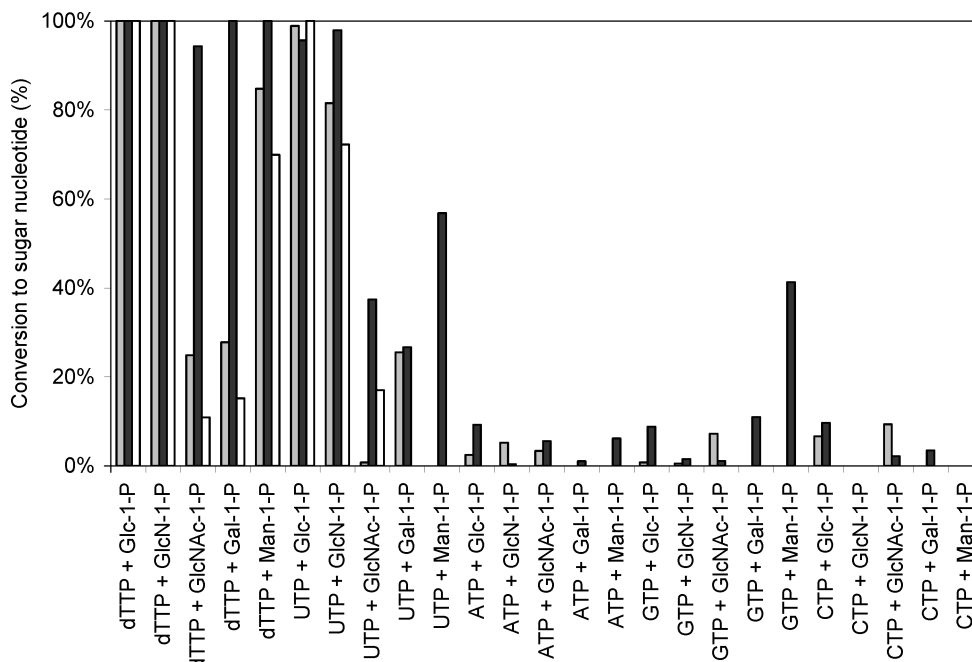


FIGURE 4: Conversions of four commercially available sugar 1-phosphates (4–7) to sugar nucleotides by the wild-type Cps2L enzyme (light gray bars), the Q24S mutant (dark gray bars) after 30 min, and the Q24D mutant (white bars) after 24 h. Conversions are based upon the consumption of nucleoside triphosphate. Reactions were catalyzed by 1 EU.

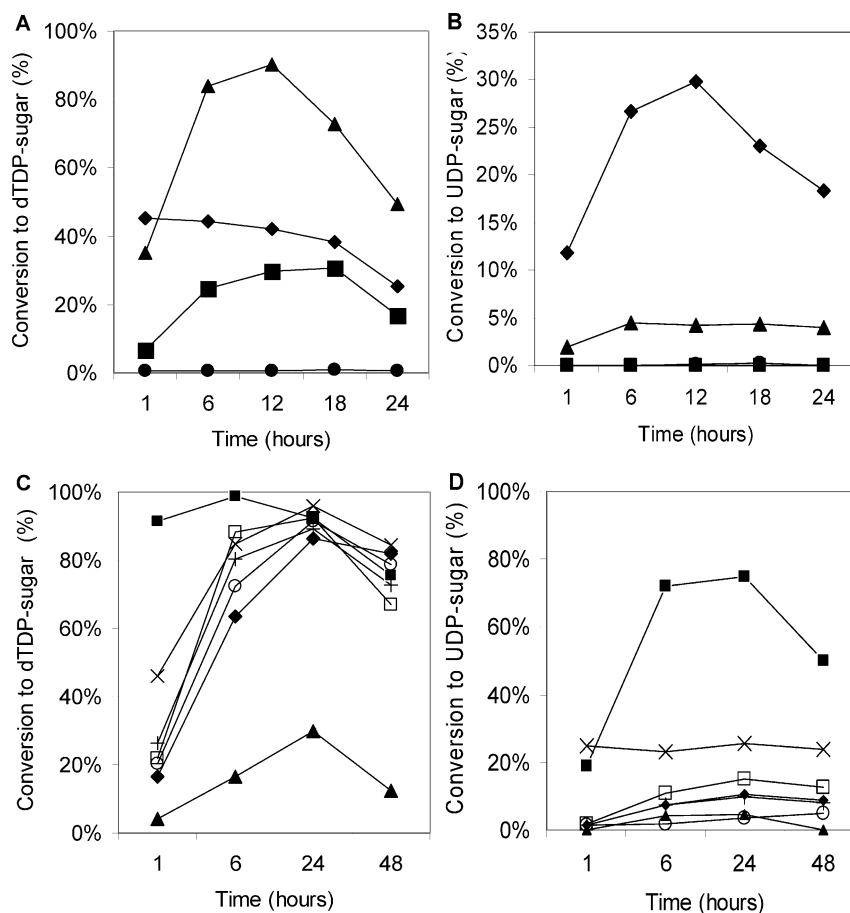


FIGURE 5: Cps2L Q24S (10 EU) conversion of synthesized sugar 1-phosphates to sugar nucleotides. Cps2L Q24S was able to convert furanosyl sugar 1-phosphates and alkylated glucose 1-phosphates to sugar nucleotides: (A and B) 8 (▲), 9 (◆), 10 (●), and 11 (■) and (C and D) 12 (■), 13 (□), 14 (▲), 15 (○), 16 (×), 17 (+), and 18 (◆). A decrease in conversion percentage results from the breakdown of the products.

1-phosphate reactivity changed between the wild-type enzyme and the mutant. With the wild-type enzyme, Araf-1-P was the most active substrate (60%, 6 h) and the mutant

could not match this level of conversion, maxing out at 50%. The mutant was most active with Gal-1-P with >90% converted to dTDP-Gal-1-P in 12 h, in stark contrast to the wild-



type enzyme that achieved only 35% conversion after 24 h. The mutant was also more effective at generating the corresponding dTDP-furanose (30%, 18 h) from 6-F-Galf-1-P than the wild-type enzyme (15%, 24 h). The reactions with the Q24S mutant, UTP, and the furanosyl 1-phosphates were more successful than those catalyzed by the wild-type enzyme. The mutant achieved 30% conversion of Araf-1-P to UDP-Araf in 12 h and <5% conversion of Galf-1-P to UDP-Galf. All conversions remain uncorrected for the starting anomeric ratio of the furanosyl 1-phosphate shown in Figure 3. Confirmation of the acceptance of only one anomeric furanosyl 1-phosphate was demonstrated by mass spectrometry. ESI-MS/MS analysis of the sugar nucleotide product provides an indication of whether 1,2-*cis* or 1,2-*trans* products have been formed, based on whether NDP or NMP is the major fragment in the enhanced product ion scan (fragmentation), respectively (33). We demonstrated previously that Cps2L was selective for only the 1,2-*cis* furanosyl 1-phosphates (29). We confirmed that the Q24S mutant produced identical products. The mass spectrometry fragmentation for 1,2-*cis* or 1,2-*trans* sugar nucleotides has been demonstrated independently by Turnock and Ferguson (34).

Catalysis by Q24S of the 3-*O*-alkylglucose 1-phosphate condensations with dTTP resulted in good conversions to product, which had been observed with the wild-type enzyme. Generally, the mutant was capable of achieving 60% conversion within 6 h, whereas the wild-type enzyme required 24 h to reach 60%. When the nucleoside triphosphate was switched to UTP, two of the alkyl substrates exhibited significantly improved activity over the results with the wild-type enzyme. 3-*O*-Methylglucose 1-phosphate was converted to >70% product in 6 h, and 3-*O*-dodecylglucose 1-phosphate was converted in 25% after 1 h. Other 3-*O*-alkylglucose 1-phosphate substrates were converted approximately 2-fold more efficiently over 24 h with the Q24S mutant relative to the wild-type enzyme; however, the highest conversion obtained was 15% at 24 h.

There is a remarkable structural similarity between the uridylyltransferase and thymidyltransferase families in prokaryotes. We compared the dTDP-Glc and UDP-Glc binding sites in the *P. aeruginosa* (17) and *Corynebacterium glutamicum* (31) enzymes (Figure 6) deduced by X-ray crystallography to compare how Nature catalyzes reactions with dTTP and UTP. The two enzymes are 22% identical (Supporting Information). The sugar nucleotide binding sites were aligned such that the base, (deoxy)ribose, and glucose functionalities of the bound product all overlapped. There were three significant amino acid substitutions in the nucleoside triphosphate binding pocket. In the active site that binds dTTP as the physiological substrate, namely, *P. aeruginosa* enzyme RmlA, residues L8, Q26, and G109 were within 4 Å of the product. In the *C. glutamicum* enzyme, the corresponding residues were P19, E36, and P141. The L8 and P19 residues are to be found at the end of a  $\beta$ -strand, the Q26 and E36 residues within a 3/10 helix, and the G109 and P141 residues in a hydrogen-bonded turn (Supporting Information). A sequence alignment including other related uridylyl- and thymidyltransferases (Supporting Information) indicates that the first two substitutions were conserved within each family, while the third site of modification, P141, was observed only in the *C. glutamicum* enzyme. In other uridylyltransferases, this third modification was frequently

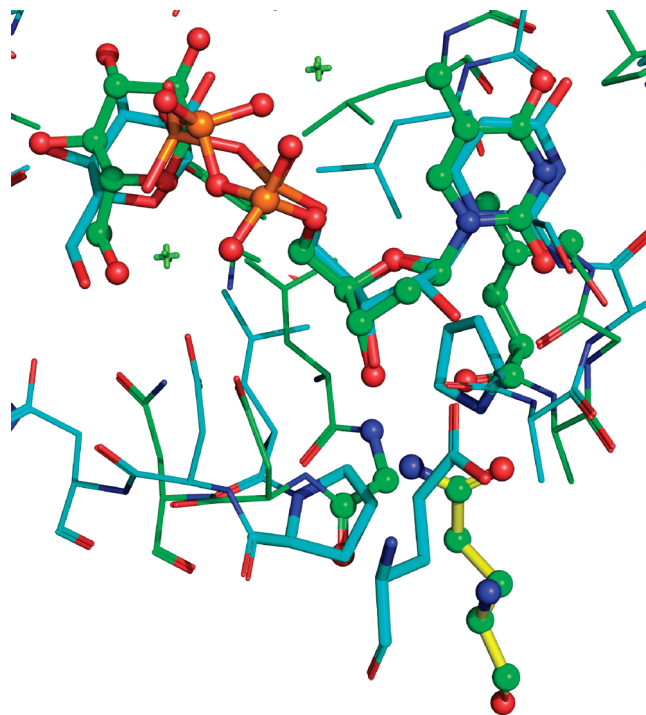


FIGURE 6: Comparison of dTDP- and UDP-nucleotidyltransferase active sites from *C. glutamicum* (PDB entry 2pe4, carbon atoms colored cyan) and *P. aeruginosa* (PDB entry 1g3l, carbon atoms colored green) showing residues within 4 Å of the sugar nucleotide. The UDP-Glc and dTDP-Glc residues were aligned using Pymol, ensuring overlap of the base, ribose, and glucose moieties of the sugar nucleotide. The only amino acid differences in the nucleoside triphosphate binding region are highlighted: ball-and-stick L8, Q26, and G109 residues of the *P. aeruginosa* enzyme and stick P19, E36, and P141 residues of the *C. glutamicum* enzyme.

a glycine residue, consistent with the thymidyltransferases. Thus, the difference between amino acid residues in the thymidyltransferase and uridylyltransferase nucleoside triphosphate binding site is limited to two substitutions: L8 with P19 and Q26 with E36.

## DISCUSSION

The initial survey using commercially available hexose 1-phosphates comparing the relative activities of the wild-type Cps2L enzyme and the Q24S mutant indicated that the mutant was significantly more active than the wild-type enzyme with respect to the formation of dTDP-sugars; this result was even more pronounced with the formation of UDP-sugars where little activity was observed with the wild-type enzyme, validating the importance of the Q24S residue in binding ribo-configured nucleoside triphosphates. This indicates that the Q24S mutation not only improves the relative activity of the enzyme with nonphysiological nucleoside triphosphates but also influences binding and catalysis of nonphysiological sugar 1-phosphates. The analysis of more structurally diverse sugar 1-phosphate substrate classes resulted in improvements in relative activity for the production of dTDP- and UDP-sugars, although without the substantial improvements observed with the commercial hexose 1-phosphates. The more sterically demanding 3-*O*-alkylglucose 1-phosphates were converted to products approximately 2-fold faster with Q24S than with the wild-type enzyme; however, this relative rate enhancement was not as significant as that observed with the commercially available

hexose 1-phosphates and can presumably be attributed to the increase in steric bulk, which likely weakens their ability to bind. The furanosyl phosphates are limited as substrates by low turnover, as determined by significantly lower  $k_{\text{cat}}$  values with the wild-type enzyme (29), and the mutation within the nucleoside triphosphate binding site is presumably insufficient for drastically enhancing reactivity with the furanosyl phosphates, although it does alter the order of reactivity. The presence of a smaller amino acid residue in the mutant active site may confer greater malleability and tolerance toward nonphysiological substrates. The Q24S mutant preferred Gal $\beta$ -1-P, whereas the wild-type enzyme preferred Ara $\beta$ -1-P. The ordered Bi-Bi mechanism widely acknowledged for this enzyme class indicates that the nucleoside triphosphate binds prior to the sugar 1-phosphate. Thus, subtle alterations of the sugar 1-phosphate binding site occur as a result of the nucleoside triphosphate binding to the Q24S mutant, leading to switches in sugar 1-phosphate specificity. Significantly, the Q24S mutation improves conversions with various deoxy- and ribo-configured nucleoside triphosphates, consistent with a less stringent nucleoside triphosphate binding site with differing potentials for hydrogen bonding to the wild-type enzyme.

The analyses of the uridylyltransferase and thymidylyltransferase crystal structures indicate that two residues within the binding site are responsible for the change in specificity between dTDP- and UDP-sugars. It is interesting that the Q24D mutation in our thymidylyltransferase, which provides the same acidic side chain functionality found in the uridylyltransferases, resulted in a mutant with a 40-fold lower relative activity. Potentially, this could be attributed to the lack of an additional methylene functionality in the aspartic acid relative to glutamic acid substitution, or the need to switch leucine for proline at the end of  $\beta$ -sheet 1. The success of the Q24S mutation in generating a catalyst able to produce dTDP-sugars and UDP-sugars appears to be a new distinct mutation within prokaryotes.

In conclusion, the Q24S mutation gave rise to an enzyme variant with improved relative activity with dTTP compared to the wild-type enzyme, by improving the production of dTDP-Gal, dTDP-GlcNAc, and dTDP-Man and also the formation of sugar nucleotides from several furanosyl 1-phosphates and 3-*O*-alkylglucose 1-phosphate derivatives. It also improved the UTP relative activity in comparison to that of the wild-type enzyme, as determined by catalysis with a broader range of sugar 1-phosphates, including the generation of UDP-GlcNAc, UDP-Man, GDP-Man, and several 3'-*O*-alkyl UDP-Glc derivatives and UDP-furanoses. Thus, the Q24S mutant (Q26, *P. aeruginosa* numbering) appears to be a new approach to generating UDP-sugars and is distinct from that used in the *C. glutamicum* uridylyltransferase and homologous uridylyltransferases in prokaryotes. Further optimization of the sugar 1-phosphate binding site of these promiscuous catalysts will facilitate production of more exotic sugar nucleotides for glycobiology studies.

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## SUPPORTING INFORMATION AVAILABLE

SDS-PAGE gel of wild-type and mutant Cps2L catalysts, retention times for sugar nucleotide products, sequence analysis of *C. glutamicum* uridylyltransferase (PDB entry 2pa4), sequence analysis of *P. aeruginosa* thymidylyltransferase (PDB entry 1g11), sequence alignment of representative prokaryotic uridylyltransferases and thymidylyltransferases, and ESI-MS/MS EPI scan data of enzyme-catalyzed production of UDP-furanoses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## REFERENCES

- Varki, A. (1993) Biological Roles of Oligosaccharides: All of the Theories are Correct. *Glycobiology* 3, 97–130.
- Dove, A. (2002) The Bittersweet Promise of Glycobiology. *Nat. Biotechnol.* 19, 913–917.
- Raetz, C. R., and Whitfield, C. (2002) Lipopolysaccharide Endotoxins. *Annu. Rev. Biochem.* 71, 635–700.
- Finlay, B. B., and Falkow, S. (1997) Common Themes in Microbial Pathogenicity Revisited. *Microbiol. Mol. Biol. Rev.* 61, 136–169.
- Thorson, J. S., Hosted, T. J., Jiang, J. Q., Biggins, J. B., and Ahlert, J. (2001) Nature's Carbohydrate Chemists: The Enzymatic Glycosylation of Bioactive Bacterial Metabolites. *Curr. Org. Chem.* 5, 139–167.
- Kuhn, H. M., Meier-Dieter, U., and Mayer, H. (1988) ECA, the Enterobacterial Common Antigen. *FEMS Microbiol. Rev.* 4, 195–222.
- Godfroid, F., Cloeckert, A., Taminiau, B., Danese, I., Tibor, A., de Bolle, X., Mertens, P., and Letesson, J. J. (2000) Genetic Organisation of the Lipopolysaccharide O-Antigen Biosynthesis Region of *Brucella melitensis* 16M (Wbk). *Res. Microbiol.* 151, 655–668.
- Rose, N. L., Completo, G. C., Lin, S. J., McNeil, M., Palcic, M. M., and Lowary, T. L. (2006) Expression, Purification, and Characterization of a Galactofuranosyltransferase Involved in *Mycobacterium tuberculosis* Arabinogalactan Biosynthesis. *J. Am. Chem. Soc.* 128, 6721–6729.
- Lecca, D., and Ceruti, S. (2008) Uracil Nucleotides: From Metabolic Intermediates to Neuroprotection and Neuroinflammation. *Biochem. Pharmacol.* 10.1016/j.bcp.12.009.
- Jiang, J. Q., Biggins, J. B., and Thorson, J. S. (2000) A General Enzymatic Method for the Synthesis of Natural and "Unnatural" UDP- and TDP-Nucleotide Sugars. *J. Am. Chem. Soc.* 122, 6803–6804.
- Truman, A. W., Huang, F., Llewellyn, N. M., and Spencer, J. B. (2007) Characterization of the Enzyme BtrD from *Bacillus circulans* and Revision of its Functional Assignment in the Biosynthesis of Butirosin. *Angew. Chem., Int. Ed.* 46, 1462–1464.
- Murrell, J. M., Liu, W., and Shen, B. (2004) Biochemical Characterization of the SgcA1  $\alpha$ -D-Glucopyranosyl-1-Phosphate Thymidylyltransferase from the Enediyne Antitumor Antibiotic C-1027 Biosynthetic Pathway and Overexpression of sgcA1 in *Streptomyces globisporus* to Improve C-1027 Production. *J. Nat. Prod.* 67, 206–213.
- Timmons, S. C., Mosher, R. H., Knowles, S. A., and Jakeman, D. L. (2007) Exploiting Nucleotidylyltransferases to Prepare Sugar Nucleotides. *Org. Lett.* 9, 857–860.
- Mizanur, R. M., Zea, C. J., and Pohl, N. L. (2004) Unusually Broad Substrate Tolerance of a Heat-Stable Archaeal Sugar Nucleotidylyltransferase for the Synthesis of Sugar Nucleotides. *J. Am. Chem. Soc.* 126, 15993–15998.
- Errey, J. C., Mukhopadhyay, B., Ravindranathan Kartha, K. P., and Field, R. A. (2004) Flexible enzymatic and chemo-enzymatic approaches to a broad range of uridine-diphospho-sugars. *Chem. Commun.*, 2706–2707.
- Zhang, Z., Tsujimura, M., Akutsu, J., Sasaki, M., Tajima, H., and Kawarabayashi, Y. (2005) Identification of an Extremely Thermostable Enzyme with Dual Sugar-1-Phosphate Nucleotidylyltransferase Activities from an Acidothermophilic Archaeon *Sulfolobus tokodaii* strain 7. *J. Biol. Chem.* 280, 9698–9705.
- Blankenfeldt, W., Asuncion, M., Lam, J. S., and Naismith, J. H. (2000) The Structural Basis of the Catalytic Mechanism and Regulation of Glucose-1-Phosphate Thymidylyltransferase (RmlA). *EMBO J.* 19, 6652–6663.

18. Zuccotti, S., Zanardi, D., Rosano, C., Sturla, L., Tonetti, M., and Bolognesi, M. (2001) Kinetic and Crystallographic Analyses Support a Sequential-Ordered Bi Bi Catalytic Mechanism for *Escherichia coli* Glucose-1-Phosphate Thymidyltransferase. *J. Mol. Biol.* 313, 831–843.
19. Thoden, J. B., and Holden, H. M. (2007) The Molecular Architecture of Glucose-1-Phosphate Uridyltransferase. *Protein Sci.* 16, 432–440.
20. Kostrewa, D., D'Arcy, A., Takacs, B., and Kamber, M. (2001) Crystal Structures of *Streptococcus pneumoniae* N-Acetylglucosamine-1-Phosphate Uridyltransferase, GlmU, in Apo Form at 2.33 Å Resolution and in Complex with UDP-N-Acetylglucosamine and Mg<sup>2+</sup> at 1.96 Å Resolution. *J. Mol. Biol.* 305, 279–289.
21. Jin, X., Ballicora, M. A., Preiss, J., and Geiger, J. H. (2005) Crystal Structure of Potato Tuber ADP-Glucose Pyrophosphorylase. *EMBO J.* 24, 694–704.
22. Steiner, T., Lamerz, A. C., Hess, P., Breithaupt, C., Krapp, S., Bourenkov, G., Huber, R., Gerardy-Schahn, R., and Jacob, U. (2007) Open and Closed Structures of the UDP-Glucose Pyrophosphorylase from *Leishmania major*. *J. Biol. Chem.* 282, 13003–13010.
23. Sivaraman, J., Sauve, V., Matte, A., and Cygler, M. (2002) Crystal Structure of *Escherichia coli* Glucose-1-Phosphate Thymidyltransferase (RffH) Complexed with dTTP and Mg<sup>2+</sup>. *J. Biol. Chem.* 277, 44214–44219.
24. Jiang, J. Q., Albermann, C., and Thorson, J. S. (2003) Application of the Nucleotidyltransferase E-p Toward the Chemoenzymatic Synthesis of dTDP-Desosamine Analogues. *ChemBioChem* 4, 443–446.
25. Thorson, J. S., Barton, W. A., Hoffmeister, D., Albermann, C., and Nikolov, D. B. (2004) Structure-Based Enzyme Engineering and its Impact on in Vitro Glycorandomization. *ChemBioChem* 5, 16–25.
26. Barton, W. A., Lesniak, J., Biggins, J. B., Jeffrey, P. D., Jiang, J. Q., Rajashankar, K. R., Thorson, J. S., and Nikolov, D. B. (2001) Structure, Mechanism and Engineering of a Nucleotidyltransferase as a First Step Toward Glycorandomization. *Nat. Struct. Biol.* 8, 545–551.
27. Barton, W. A., Biggins, J. B., Jiang, J., Thorson, J. S., and Nikolov, D. B. (2002) Expanding Pyrimidine Diphosphosugar Libraries Via Structure-Based Nucleotidyltransferase Engineering. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13397–13402.
28. Huestis, M. P., Aish, G. A., Hui, J. P., Soo, E. C., and Jakeman, D. L. (2008) Lipophilic Sugar Nucleotide Synthesis by Structure-Based Design of Nucleotidyltransferase Substrates. *Org. Biomol. Chem.* 6, 477–484.
29. Timmons, S. C., Hui, J. P., Pearson, J. L., Peltier, P., Daniellou, R., Nugier-Chauvin, C., Soo, E. C., Syvitski, R. T., Ferrières, V., and Jakeman, D. L. (2008) Enzyme-Catalyzed Synthesis of Furanosyl Nucleotides. *Org. Lett.* 10, 161–163.
30. Moretti, R., and Thorson, J. S. (2007) Enhancing the Latent Nucleotide Triphosphate Flexibility of the Glucose-1-Phosphate Thymidyltransferase RmlA. *J. Biol. Chem.* 282, 16942–16947.
31. Thoden, J. B., and Holden, H. M. (2007) Active Site Geometry of Glucose-1-Phosphate Uridyltransferase. *Protein Sci.* 16, 1379–1388.
32. Beaton, S. A., Huestis, M. P., Sadeghi-Khomami, A., Thomas, N. R., and Jakeman, D. L. (2008) Enzyme-Catalyzed Synthesis of Isosteric Phosphono-Analogues of Sugar Nucleotides. (submitted for publication).
33. Wolucka, B. A., Rush, J. S., Waechter, C. J., Shibaev, V. N., and de Hoffmann, E. (1998) An Electrospray-Ionization Tandem Mass Spectrometry Method for Determination of the Anomeric Configuration of Glycosyl 1-Phosphate Derivatives. *Anal. Biochem.* 255, 244–251.
34. Turnock, D. C., and Ferguson, M. A. J. (2007) Sugar Nucleotide Pools of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. *Eukaryotic Cell* 6, 1450–1463.

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