DOI: 10.1002/cbic.200500183

A Practical Enzymatic Synthesis of UDP Sugars and NDP Glucoses

Jungdon Bae,[a, b] Kwang-Ho Kim,[a] Dooil Kim,[a, c] Yongseok Choi,[a] Joong Su Kim,[a] Sukhoon Koh,[a] Suk-In Hong,[d] and Dae-Sil Lee*[a]

Nucleoside diphosphate (NDP) sugar pyrophosphorylases (N_Ps) catalyze the bioconversion of sugar-1-phosphates (Su1Ps) and nucleoside triphosphates (NTPs) to NDP sugars.[1] Various glycosyltransferases[2] specifically transfer these activated monosaccharide residues onto a free hydroxyl or amino group on an acceptor molecule to synthesize bioactive metabolites such as glycogen, trehalose, lipopolysaccharides, and peptidoglycans, which are present in the bacterial cell wall.[3] Furthermore, many pharmaceutically relevant natural products are glycosylated secondary metabolites, which are thought to have potential for the development of novel glycorandomization therapeutics.[4] Although chemical syntheses of a few NDP sugars have been reported by various groups,[5] the methods used were complicated by the laborious maneuvering of protecting groups in order to achieve regioselectivity. By contrast, N_Ps from natural sources catalyze regioselective phosphorylation by modulation of the metabolic intermediates. For these reasons, a variety of in vitro synthetic routes to NDP sugars by a salvage or a de novo pathway have been designed and implemented.[6–8] In view of the synthetic availability of high-energy donor NDP sugars for glycosyltransferases, we report the synthesis of UDP sugars and NDP glucoses by recombinant *Thermus caldophilus* GK24 (*Tca*) UDP-sugar pyrophosphorylase (U_P) and the substrate specificity of the enzyme toward various NTPs and Su1Ps.

The *Tca usp* gene (GeneBank accession no.: AAV80705), which encodes U_P, was cloned and inserted into the pKK223-3 vector to construct plasmid pGLM.[9] The tetrameric U_P protein was overexpressed at an optimal temperature of 70 °C in *Escherichia coli* MV1184 harboring pGLM (see the Supporting Information), and purified by heat precipitation and one-step anion-exchange column chromatography (Figure 1).[10] The presence of Mg^{2+} and Mn^{2+} at an appropriate concentration was required for *Tca* U_P activity; Co^{2+} and Ni^{2+} were less effective (see the Supporting Information). A thermostability test showed that *Tca* U_P was very stable at 70 °C for 1 h (see the Supporting Information).

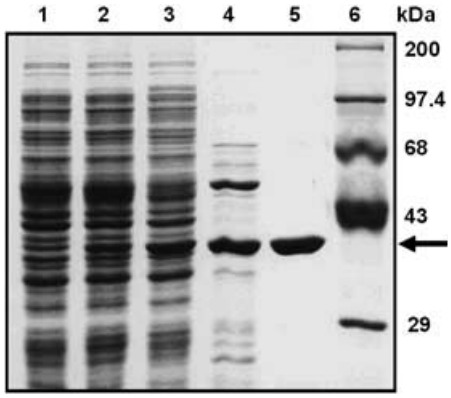


Figure 1. SDS-PAGE analysis of *Tca* U_P purified from *E. coli* MV1184/pGLM. Lane 1: *E. coli* MV1184 cell extract, lane 2: IPTG-uninduced *E. coli* MV1184/pGLM cell extract, lane 3: IPTG-induced *E. coli* MV1184/pGLM cell extract, lane 4: heat-precipitation extract, lane 5: DEAE-sephacel chromatography, lane 6: size marker. Myosin (200 kDa), phosphorylase b (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). The arrow indicates the position of *Tca* U_P.

Forty-five enzymatic reactions with nine Su1Ps[11] were initiated by the addition of five NTPs (**1, 12**–**15**) at 70 °C (Scheme 1 and Table S2 in the Supporting Information).[12] The reactions were evaluated by HPLC.[13] *Tca* U_P showed broad specificity toward five types of Su1P (**2**–**6**) with UTP (**1**). Activity toward the synthesis of **7** (specific activity, 158.7 U mg^{-1}) was the highest by *Tca* U_P, followed by **10** (116.9 U mg^{-1}), **9** (114.9 U mg^{-1}), and **8** (76.4 U mg^{-1}), which were formed from **5**, **4**, and **3**, respectively. The activity of **11** formed from **6** was the lowest (66.4 U mg^{-1}) in the presence of compound **1**. In the *Tca* U_P-catalyzed reaction of four types of NTP (**12**–**15**) with Glc1P (**6**), all of the NDP glucoses (**16**–**19**) were formed (Scheme 1B). The specific activity was as follows: **19** (142.8 U mg^{-1}), **17** (128.8 U mg^{-1}), **16** (56.4 U mg^{-1}), and **18** (54.4 U mg^{-1}). These results also showed that *Tca* U_P (as pea sprout U_P[8c]) effectively catalyzed the formation of all the NDP glucoses (**16**–**19**) from **6** in the presence of purine (**12** and **13**) and pyrimidine nucleotides (**1**, **14**, and **15**). However, in the other Su1Ps, such as GlcN1P, Fuc1P, Fru1P, and Mal1P, no product was formed in the presence of any NTP by the enzyme reaction. Moreover, *Tca* U_P reaction with Su1P (**2**-**5**) and NTPs (**12**–**15**) other than UTP (**1**) failed to convert to the corresponding NDP sugars (Table S2 in the Supporting Information). In comparison with the substrate specificity of other N_Ps, *Salmonella enterica* LT2 glucose-1-phosphate thymidylyltransferase (E_P), which has broad sugar specificity over TTP and UTP, is more specific for the pyrimidine TDP sugars than UDP sugars.[6g,i] Sugar nucleotidyltransferase (UDPG-PPase) from an archaeal *Pyrococcus furiosus* DSM 3638

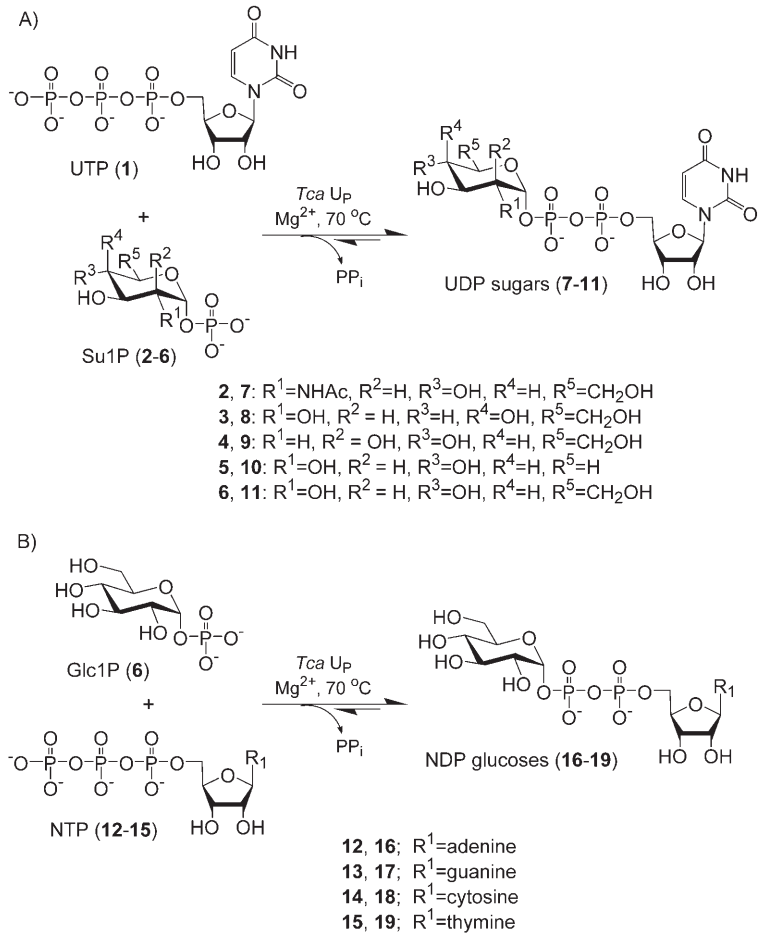
[a] *Dr. J. Bae, K.-H. Kim, D. Kim, Dr. Y. Choi, Dr. J. S. Kim, Dr. S. Koh, Dr. D.-S. Lee*
Genome Research Center
Korea Research Institute of Bioscience and Biotechnology
Yuseong, Daejeon 305-333 (Korea)
Fax: (+82) 42-860-4597
E-mail: daesilee@kribb.re.kr

[b] *Dr. J. Bae*
School of Life sciences and Biotechnology, Korea University
Seoul 136-701 (Korea)

[c] *D. Kim*
Department of Biomicrosystem Technology, Korea University
Seoul 136-701 (Korea)

[d] *Prof. S.-I. Hong*
Department of Chemical and Biological Engineering, Korea University
Seoul 136-701 (Korea)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: experimental procedures for cloning, expression, and characterization of Tca U_P *and* ^1H *and* ^{13}C *NMR data for* ***11***–***19***.



Scheme 1. *Tca* U_P-catalyzed synthesis of A) UDP sugars and B) NDP glucoses.

(*Pfu*) has higher activity toward UTP than TTP with broad sugar specificity; however, a purine nucleotide, ATP, was not accepted by the enzyme.[8b] Our results indicate that the recombinant U_P can form not only several UDP sugars (**7**–**11**) from Su1P (**2**–**6**) in the presence of UTP (**1**), but also NDP glucoses (**16**–**19**) in the presence of both pyrimidine and purine nucleotides (**12**–**15**), thus proving a novel catalytic behavior.

For the synthesis of nucleotide sugars on a preparative scale, each enzymatic reaction was performed at 70 °C for 1 h (Table 1). The U_P products were readily purified by two steps of silica gel chromatography. The conversion of **7** (89.9%) was the highest among the synthetic products, as given in Table 1, and that of **18** (17.1%) was the lowest. Identification of all the products was based on ^{1}H and ^{13}C NMR spectra (see the Supporting Information). These results are in agreement with the above substrate-specificity assays. Overall, the conversion of UDP sugars is relatively higher than that of NDP glucoses under given conditions. As far as we know, NDP glucoses (**11**, **16**, **17**, and **19**) in the genus *Thermus* will donate glucose molecules in preference to the donation of the glucose-6-phosphate of trehalose-6-phosphate synthase in the trehalose synthetic pathway for osmoadaptation.[14] Compound **7** is used as an essential precursor for the biosynthetic pathways of peptidoglycan in the bacterium *T. caldophilus* in our ongoing study (data not shown). Additional *Tca* N_P, ADP-glucose pyrophosporylase[15] is only highly specific toward **16** for the synthesis of glycogen as the major carbohydrate reserve. Recently, it was reported that the repeated polysaccharide in *Thermus thermophilus* HB8 contains several monosaccharide units, such as mannose, galactose, glucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine.[16] It is possible that these sugars are closely related to the in vivo function of *Tca* U_P.

To understand the molecular-level interactions at the binding site of the *Tca* U_P substrates, *Tca* U_P was compared with seven crystal structures (Figure 2) for sequence homology among various N_P enzymes. It appears that three amino acid residues (G9, R13, and K23) in the N-terminal domain of *Tca* U_P interact with nucleosides and are well conserved.[7a,17] Substitution of other amino acid residues (R10, L14–P22) might afford *Tca* U_P broad NTP specificity. It is especially proposed that R217 (N227 in two GlmUs) stabilizes several nucleoside substrates through hydrogen bonding. In GlmU from *E. coli*, a catalytic domain composed of six amino acids (T82, Y139, G140, E154, N169, and Y197) is implicated in the binding of GlcNAc1P. Among the residues, T82, E154, and Y197 interact directly with GlcNAc1P rather than with Glc1P; this results in the exclusive formation of UDP-GlcNAc.[17a] The corresponding residues in *S. enterica* are L89, Y146, G147, E162, V193, and E199. Notably, the *S. enterica* L89T E_P mutant enzyme[7b] enhances the promiscuity at position C-2 of the substrates; this might decrease the steric hindrance of Su1P. The *S. enterica* E162D E_P mutant enzyme[7a] fails to synthesize any NDP sugar. These amino acids are replaced by L81, F136, G137, K152, V162, and E188 in *Tca*

Table 1. Conversion and purification yield of nine NDP sugars synthesized by *Tca* U_P.[a]

Substrate NTP	Su1P	Product	Conv.[b] [%]	Purified yield [mg (%)]
UTP (**1**)	GlcNAc1P (**2**)	UDP-GlcNAc (**7**)	89.9	41.1 (63.1)
UTP	Gal1P (**3**)	UDP-Gal (**8**)	39.1	14.0 (22.9)
UTP	Man1P (**4**)	UDP-Man (**9**)	66.8	27.9 (45.7)
UTP	Xyl1P (**5**)	UDP-Xyl (**10**)	58.3	25.6 (41.9)
UTP	Glc1P (**6**)	UDP-Glc (**11**)	31.6	9.7 (15.3)
ATP (**12**)	Glc1P (**6**)	ADP-Glc (**16**)	27.9	11.0 (18.2)
GTP (**13**)	Glc1P	GDP-Glc (**17**)	39.1	20.2 (33.2)
CTP (**14**)	Glc1P	CDP-Glc (**18**)	17.1	7.8 (12.8)
TTP (**15**)	Glc1P	TDP-Glc (**19**)	44.1	24.0 (44.8)

[a] The reaction mixture (50 mL), containing the *Tca* U_P (11.1 U mL^{-1}), Tris-HCl buffer (25 mM, pH 8.0), NTP (2 mM), Su1P (2 mM), and $MgCl_2$ (8 mM), was incubated at 70 °C for 1 h. [b] Conversion = [NDP sugar/NTP × 100] was calculated from the enzymatic reaction with Su1P (2 mM) and NTP (2 mM) at 70 °C for 1 h. HPLC retention times [min] for each compound at 254 nm were as follows: **7**, 13.0; **8**, 12.7; **9**, 12.5; **10**, 14.2; **11**, 12.7; **16**, 14.4; **17**, 12.8; **18**, 10.7; **19**, 13.0; UMP, 4.8; UDP, 13.7; **1**, 25.2; AMP, 7.1; ADP, 16.9; **12**, 25.2; GMP, 5.5; GDP, 15.0; **13**, 25.1; CMP, 4.3; CDP, 12.1; **14**, 23.7; TMP, 6.0; TDP, 15.2; **15**, 24.8.

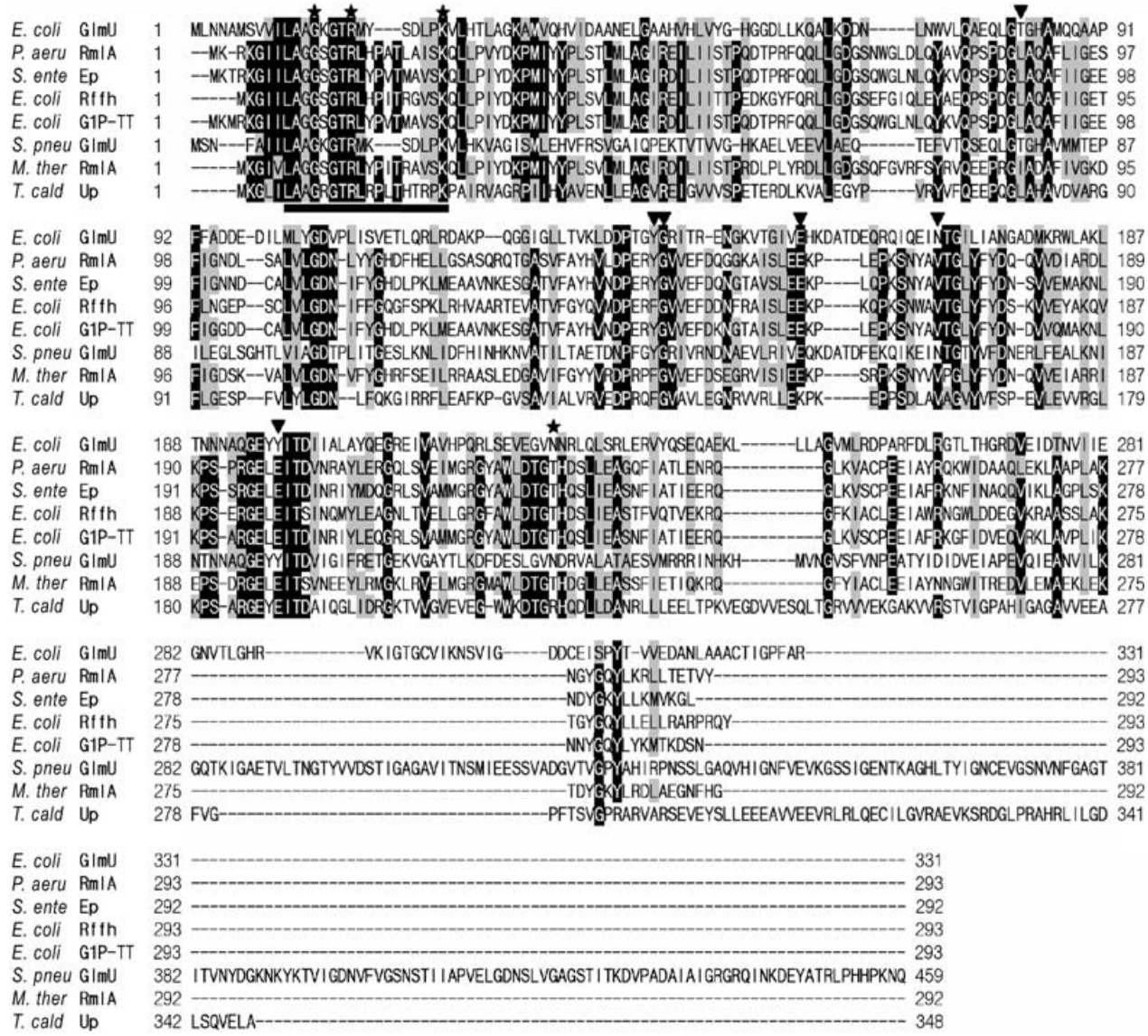


Figure 2. Multiple sequence alignment of *T. caldophilus* U_P with *E. coli N*-acetylglucosamine 1-phosphate uridyltransferase (*E. coli* GlmU; PDB code, 1FXJ; sequence similarity, 29.9%) for the precursor (**7**) synthesis of peptidoglycan and lipopolysaccharides, *Pseudomonas aeruginosa* glucose-1-phosphate thymidylyltransferase (*P. aeru* RmlA; 1G23; 36.7%), the first enzyme to form dTDP-L-rhamnose from **19** in the cell wall, *S. enterica* E_P (*S. ente* E_P; 1IIN; 38.6%) with unusual promiscuity toward various pyrimidine NDP sugars containing **7**–**9**, **11** and **19** in this report, *E. coli* glucose-1-phosphate thymidylyltransferase (*E. coli* Rffh; 1MC3; 37.8%), *E. coli* glucose-1-phosphate thymidylyltransferase (*E. coli* G1P-TT; 15H5; 38.6%), *Streptococcus pneumoniae* GlmU (*S. pneu* GlmU; 1HMO; 26.6%), and *Methanobacterium thermoautotrophicum* RmlA (*M. them* RmlA; 1LVW; 36%). * indicates possible nucleotide-binding residues. ▼ corresponds to the amino acid residues implicated in the binding of sugar. The underlined $L(X)_2GXGT(X)_2R(X)_6PK$ motif (PROSITE database; http://www.expasy.org) is a putative pyrophosphorylase consensus domain.

U_P. The three residues L81, K152, and E188 might play an important role in interacting with C-2 of Su1P. The positively charged residue K152 in *Tca* U_P is possibly more electrostatically coupled to the sugar O-2 or O-3 atom than the negatively charged residue E162 of *S. enterica* E_P; this would affect the sugar specificity of the enzyme. Residue T190 in *Tca* U_P might also be important for the tolerance of bulky *N*-acetyl group at C-2 of Su1P, based on the result of another *S. enterica* E_P variant (T201A),[7a] which converts GlcNAc1P and dTTP to dTDP-GlcNAc with high activity.

In conclusion, practical and efficient syntheses of UDP sugars and NDP glucoses by using a single recombinant enzyme with high thermostability have been demonstrated. When compared with most mesophile-driven N_P with narrowed substrate specificity, *Tca* U_P has broad substrate specificity toward purine and pyrimidine nucleotides as well as broad sugar specificity over UTP. This highlights the potential of multifunctional U_P in vitro from the thermophilic bacterium. More detailed biochemical and structural analysis toward its substrate specificity will be addressed in further studies.

Acknowledgements

This work was supported by a grant from the KRIBB Research Initiative Program, Korea.

Keywords: enzyme catalysis · glycosyltransferases · nucleotide sugars · *Thermus caldophilus*

[1] a) L. Elling, *Adv. Biochem. Eng./Biotechnol.* **1997**, *58*, 89; b) T. Bülter, L. Elling, *Glycoconjugate J.* **1999**, *16*, 147.
[2] a) Y. Ichikawa, G. C. Look, C. H. Wong, *Anal. Biochem.* **1992**, *202*, 215; b) D. Maskell, C. R. Raetz, P. D. Rick, *Trends Microbiol.* **1996**, *4*, 495; c) M. M. Palcic, *Curr. Opin. Biotechnol.* **1999**, *10*, 616.
[3] a) J. H. Ko, C. H. Kim, D. S. Lee, Y. S. Kim, *Biochem. J.* **1996**, *319*, 977; b) N. Parajuli, D. S. Lee, H. C. Lee, K. Liou, J. K. Sohng, *Biotechnol. Lett.* **2004**, *26*, 437.
[4] a) J. S. Thorson, W. A. Barton, D. Hoffmeister, C. Albermann, D. B. Nikolov, *ChemBioChem* **2004**, *5*, 16; b) J. Yang, D. Hoffmeister, L. Liu, X. Fu, J. S. Thorson, *Bioorg. Med. Chem.* **2004**, *12*, 1577; c) J. S. Thorson, E. L. Sievers, J. Ahlert, E. Shepard, R. E. Whitwam, K. C. Onwueme, M. Ruppen, *Curr. Pharm. Des.* **2000**, *6*, 1841.
[5] a) S. Roseman, J. J. Distler, J. G. Moffatt, H. G. Khorana, *J. Am. Chem. Soc.* **1961**, *83*, 659; b) D. M. Carlson, R. G. Hansen, *J. Biol. Chem.* **1962**, *237*, 1260; c) J. E. Heidlas, W. J. Lees, P. Pale, G. M. Whitesides, *J. Org. Chem.* **1992**, *57*, 146; d) M. Arlt, O. Hindsgaul, *J. Org. Chem.* **1995**, *60*, 14; e) V. Wittmann, C. H. Wong, *J. Org. Chem.* **1997**, *62*, 2144; f) S. Hanessian, P. P. Lu, H. Ishida, *J. Am. Chem. Soc.* **1998**, *120*, 13296; g) Yu. E. Tsvetkov, A. V. Nikolaev, *J. Chem. Soc. Perkin Trans. 1* **2000**, 889; h) C. L. F. Meyers, R. F. Borch, *Org. Lett.* **2001**, *3*, 3765; i) A. S. Marlow, L. L. Kiessling, *Org. Lett.* **2001**, *3*, 2517; j) C. Ernst, W. Klaffke, *J. Org. Chem.* **2003**, *68*, 5780.
[6] a) E. P. Anderson, E. S. Maxwell, R. M. Burton, *J. Am. Chem. Soc.* **1959**, *81*, 6514; b) C. H. Wong, S. L. Haynie, G. M. Whitesides, *J. Org. Chem.* **1982**, *47*, 5416; c) E. S. Simon, S. Grabowski, G. M. Whitesides, *J. Org. Chem.* **1990**, *55*, 1834; d) J. E. Heidlas, K. W. Williams, G. M. Whitesides, *Acc. Chem. Res.* **1992**, *25*, 307; e) J. E. Heidlas, W. J. Lees, G. M. Whitesides, *J. Org. Chem.* **1992**, *57*, 152; f) A. Zervosen, A. Stein, H. Adrian, L. Elling, *Tetrahedron* **1996**, *52*, 2395; g) J. Jiang, J. B. Biggins, J. S. Thorson, *J. Am. Chem. Soc.* **2000**, *122*, 6803; h) M. Pauly, A. Porchia, C. E. Olsen, K. J. Nunan, H. V. Scheller, *Anal. Biochem.* **2000**, *278*, 69; i) J. Jiang, J. B. Biggins, J. S. Thorson, *Angew. Chem.* **2001**, *113*, 1550; *Angew. Chem. Int. Ed.* **2001**, *40*, 1502; j) M. Bar-Peled, C. L. Griffith, T. L. Doering, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 12003; k) X. Ma, J. Stöckigt, *Carbohydr. Res.* **2001**, *333*, 159; l) J. Shao, J. Zhang, J. Naháłka, P. G. Wang, *Chem. Commun.* **2002**, 2586; m) Z. Liu, J. Zhang, X. Chen, P. G. Wang, *ChemBioChem* **2002**, *3*, 348.
[7] a) W. A. Barton, J. Lesniak, J. B. Biggins, P. D. Jeffrey, J. Jiang, K. R. Rajashankar, J. S. Thorson, D. B. Nikolov, *Nat. Struct. Biol.* **2001**, *8*, 545; b) W. A. Barton, J. B. Biggins, J. Jiang, J. S. Thorson, D. B. Nikolov, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 13397; c) J. S. Thorson, W. A. Barton, D. Hoffmeister, C. Albermann, D. B. Nikolov, *ChemBioChem* **2004**, *5*, 16.
[8] a) J. S. Kim, S. Koh, H. J. Shin, D. S. Lee, S. Y. Lee, *Biotechnol. Appl. Biochem.* **1999**, *29*, 11; b) R. M. Mizanur, C. J. Zea, N. L. Pohl, *J. Am. Chem. Soc.* **2004**, *126*, 15993; c) T. Kotake, D. Yamaguchi, H. Ohzono, S. Hojo, S. Kaneko, H. K. Ishida, Y. Tsumuraya, *J. Biol. Chem.* **2004**, *279*, 45728.
[9] The *Tca usp* gene was obtained from the genome data by shot-gun sequencing, and compared with the reported 15 N-terminal amino acid residues (MKGLILAAGRGTRLR) of purified U_P, which was amplified from *Tca* genomic DNA by a polymerase chain reaction.
[10] The transformed cells, harvested by centrifugation, were resuspended in buffer A (25 mL, 20 mM Tris-HCl, pH 7.0, 1 mM β-mercaptoethanol), disrupted by sonication, and centrifuged. The supernatant was heat-precipitated at 80 °C for 20 min and filtered, then loaded onto a DEAE-Sephacel column (2×20 cm—after equilibration with buffer A, the enzyme was eluted by a linear gradient of 0–0.3 M KCl at 1 mL min^{-1} on a FPLC system), and pooled, concentrated, and dialyzed with buffer A.
[11] Glc1P, glucose-1-phosphate; Gal1P, galactose-1-phosphate; GlcNAc1P, *N*-acetylglucosamine-1-phosphate; Man1P, mannose-1-phosphate; Xyl1P, xylose-1-phosphate, GlcN1P, glucosamine-1-phosphate; Fuc1P, fucose-1-phosphate; Fru1P, fructose-1-phosphate; Mal1P, maltose-1-phosphate.
[12] The standard assay mixture, containing Tris-HCl (50 mM, pH 8.0), $MgCl_2$ (8 mM), Su1P (5 mM), and NTP (5 mM), was incubated at 70 °C for 10 min in a final volume of 50 μL. One unit of the enzyme is defined as the amount of enzyme catalyzing the formation of 1 μmol of UDP-GlcNAc per min at 70 °C.
[13] The HPLC conditions were described with sight modification in ref. [8a]. Assays of the recombinant *Tca* U_P were measured by the amount of NDP sugar formed from NTP and Su1P by using HPLC (Beckman Instruments, Palo Alto, CA). A Hypersil ODS C_{18} reversed-phase column was used (3 μm, 4.6×250 mm; Phenomenex, Torra, CA), with tetrabutylammonium chloride as ion-pair reagent. The column was equilibrated with 90% (*v*/*v*) solvent A (12.2 mM tetrabutylammonium chloride in 10.2 mM KH_2PO_4, 5% acetonitrile, pH 4.0) and 10% (*v*/*v*) solvent B (70% (*v*/*v*) acetonitrile in solvent A). The reaction products were eluted on the column with a gradient of solvent B in solvent A (10–90% solvent B for 15 min, then 90–100% solvent B for 1 min). Standard samples (NMP, NDP, NTP, and NDP sugars) were prepared at concentrations of 0.02–0.5 mM. Peaks were identified from their retention times, obtained from the absorbance at 254 nm at 1 mL min^{-1}.
[14] J. H. Ko, C. H. Kim, D. S. Lee, Y. S. Kim, *Biochem. J.* **1996**, *319*, 977.
[15] A. Silipo, A. Molinaro, C. De Castro, R. Ferrara, I. Romano, B. Nicolaus, R. Lanzetta, M. Parrilli, *Eur. J. org. Chem.* **2004**, 5047.
[16] Z. Silva, S. Alarico, M. S. da Costa, *Extremophiles* **2005**, *9*, 29.
[17] a) K. Brown, F. Pompeo, S. Dixon, D. Mengin-Lecreulx, C. Cambillau, Y. Bourne, *EMBO J.* **1999**, *18*, 4096; b) W. Blankenfeldt, M. Asuncion, J. S. Lam, J. H. Naismith, *EMBO J.* **2000**, *19*, 6652; c) G. Sulzenbacher, L. Gal, C. Peneff, F. Fassy, Y. Bourne, *J. Biol. Chem.* **2001**, *276*, 11844; d) D. Kostrewa, A. D'Arcy, B. Takacs, M. Kamber, *J. Mol. Biol.* **2001**, *305*, 279; e) S. Zuccotti, D. Zanardi, C. Rosano, L. Sturla, M. Tonetti, M. Bolognesi, *J. Mol. Biol.* **2001**, *313*, 831.

Received: April 28, 2005
Published online on October 4, 2005

