



Increasing the Thermostable Sugar-1-Phosphate Nucleotidyltransferase Activities of the Archaeal ST0452 Protein through Site Saturation Mutagenesis of the 97th Amino Acid Position

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ABSTRACT The ST0452 protein is a bifunctional protein exhibiting sugar-1-phosphate nucleotidyltransferase (sugar-1-P NTase) and amino-sugar-1-phosphate acetyltransferase activities and was isolated from the thermophilic archaeon *Sulfolobus tokodaii*. Based on the previous observation that five single mutations increased ST0452 sugar-1-P NTase activity, nine double-mutant ST0452 proteins were generated with the intent of obtaining enzymes exhibiting a further increase in catalysis, but all showed less than 15% of the wild-type *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase (GlcNAc-1-P UTase) activity. The Y97A mutant exhibited the highest activity of the single-mutant proteins, and thus site saturation mutagenesis of the 97th position (Tyr) was conducted. Six mutants showed both increased GlcNAc-1-P UTase and glucose-1-phosphate uridyltransferase activities, eight mutants showed only enhanced GlcNAc-1-P UTase activity, and six exhibited higher GlcNAc-1-P UTase activity than that of the Y97A mutant. Kinetic analyses of three typical mutants indicated that the increase in sugar-1-P NTase activity was mainly due to an increase in the apparent k_{cat} value. We hypothesized that changing the 97th position (Tyr) to a smaller amino acid with similar electronic properties would increase activity, and thus the Tyr at the corresponding 103rd position of the *Escherichia coli* GlmU (*EcGlmU*) enzyme was replaced with the same residues. The Y103N mutant *EcGlmU* showed increased GlcNAc-1-P UTase activity, revealing that the Tyr at the 97th position of the ST0452 protein (103rd position in *EcGlmU*) plays an important role in catalysis. The present results provide useful information regarding how to improve the activity of natural enzymes and how to generate powerful enzymes for the industrial production of sugar nucleotides.

IMPORTANCE It is typically difficult to increase enzymatic activity by introducing substitutions into a natural enzyme. However, it was previously found that the ST0452 protein, a thermostable enzyme from the thermophilic archaeon *Sulfolobus tokodaii*, exhibited increased activity following single amino acid substitutions of Ala. In this study, ST0452 proteins exhibiting a further increase in activity were created using a site saturation mutagenesis strategy at the 97th position. Kinetic analyses showed that the increased activities of the mutant proteins were principally due to increased apparent k_{cat} values. These mutant proteins might suggest clues regarding the mechanism underlying the reaction process and provide very important information for the design of synthetic improved enzymes, and they can be used as power-

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ful biocatalysts for the production of sugar nucleotide molecules. Moreover, this work generated useful proteins for three-dimensional structural analysis clarifying the processes underlying the regulation and mechanism of enzymatic activity.

KEYWORDS *Sulfolobus tokodaii*, double mutants, site-directed mutagenesis, site saturation mutagenesis, sugar-1-phosphate nucleotidyltransferase, the ST0452 protein, thermostable enzyme

The polymeric structures of carbohydrates play important roles in their host organisms, such as providing separation between the internal and external environment of the cell, obtaining and storing energy, and recognizing foreign cells. Polymeric carbohydrates are synthesized only from sugar nucleotides, which are an activated form of sugar molecules. Sugar nucleotides are synthesized from sugar-1-phosphate (sugar-1-P [e.g., GlcNAc-1-P]) and nucleoside triphosphate (NTP) through the action of sugar-1-phosphate nucleotidyltransferase (sugar-1-P NTase). Similar enzymes have been isolated from many organisms, including eukaryotes, bacteria, and thermophilic archaea. *N*-Acetyl-D-glucosamine is one of the most important sugar molecules because it is usually directly attached to membrane proteins or phospholipids and acts as an anchor to which polymeric carbohydrate structures are subsequently added.

The *Archaea* were established as a domain of microorganisms separate from *Bacteria* on the basis of their 16S rRNA gene sequences approximately 40 years ago (1). A thermophilic crenarchaeon, *Sulfolobus tokodaii* strain 7, was isolated from Beppu Hot Springs in Japan in the 1980s (2). *S. tokodaii* can grow optimally under acidic (pH 2 to 3) and aerobic conditions at temperatures between 70 and 80°C. The genome of this microorganism has been sequenced (3), and several genes were predicted to encode sugar-1-P NTase enzymes. Similarity analysis predicted that one of these genes, the ST0452 gene, likely encodes a glucose-1-phosphate thymidyltransferase (Glc-1-P TTase), and the activity of the protein product of this gene was therefore characterized (4). Our investigations to date have shown that the ST0452 protein exhibits high thermostability, Glc-1-P TTase activity as predicted, and unexpectedly high *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase (GlcNAc-1-P UTase) activity (Fig. 1) as well as amino-sugar-1-phosphate acetyltransferase (amino-sugar-1-P AcTase) activity (4, 5). Comparison of the activities of the ST0452 protein to those of similar enzymes from bacteria showed that both the apparent K_m and k_{cat} values of the ST0452 GlcNAc-1-P UTase activity were smaller than those of *Escherichia coli* GlnU (*EcGlnU*) enzymes (6, 7). These findings indicate that the archaeal ST0452 protein can accept a low concentration of substrate but that its turnover rate is lower than that of the *EcGlnU* enzyme (7).

In previous work (6), we attempted to improve the low turnover rate of ST0452 GlcNAc-1-P UTase activity by Ala substitution of amino acid residues predicted to be important for its activity in the reaction center. Eleven mutant ST0452 proteins were constructed, of which five exhibited higher sugar-1-P NTase activities than the wild-type enzyme (6), but these activities were still lower than those of similar bacterial enzymes. The construction of a variant protein exhibiting higher activity would be useful both for basic scientific applications and for the industrial production of UDP-GlcNAc. In the present study, we therefore attempted to construct mutant ST0452 proteins with enhanced activity using two independent strategies: introduction of a double mutant and site saturation mutagenesis at the 97th position (Tyr) of the ST0452 protein. Finally, we succeeded in constructing the mutant ST0452 proteins with high sugar-1-P NTase activity. The kinetic analysis results presented here may be useful for constructing enzymes designed with improved activity applicable to the industrial production of specific sugar nucleotide molecules.

RESULTS

Analyses of the double-mutant ST0452 proteins. We previously reported that five single-substitution mutant ST0452 proteins, G9A, T80A, Y97A, Y97F, and K147A, exhibited increased GlcNAc-1-P UTase activity (6). However, the activities of these mutant

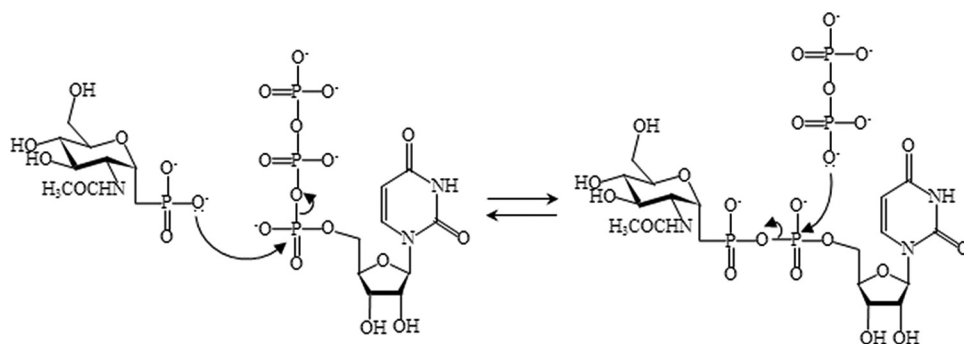


FIG 1 The reaction scheme of the sugar-1-P UTase. UTP is shown as a nucleoside triphosphate substrate.

ST0452 proteins measured at 80°C were still lower than the activity of *EcGlmU*, a similar enzyme from *E. coli*, measured at 37°C (6, 7). We anticipated that combinations of two single mutations in the ST0452 protein might significantly increase activity, and thus double-mutant ST0452 proteins were constructed in the current study to evaluate this expectation.

Nine mutant ST0452 expression vectors with independent double-mutant ST0452 genes were introduced into *E. coli* cells, and the proteins were easily expressed. After treatment at 80°C for 20 min, all double-mutant ST0452 proteins remained in the soluble fraction (data not shown), revealing that they maintained thermostabilities similar to the thermostability of the parental ST0452 protein. The GlcNAc-1-P UTase activities of the nine double-mutant ST0452 proteins were analyzed according to the protocol described below in Materials and Methods; as shown in Table 1, their specific activities were drastically lower than those of the single-mutant and wild-type ST0452 proteins and were approximately 15% or less than the GlcNAc-1-P UTase activity exhibited by the wild-type ST0452 protein (Fig. 2). These results indicated that the introduction of two single mutations decreased the activity of the ST0452 protein and that an alternative approach was required.

Analyses of mutant ST0452 proteins generated using a site saturation mutagenesis strategy at the 97th position (Tyr). The introduction of a double mutation into the ST0452 protein decreased GlcNAc-1-P UTase activity compared to that with single mutations, as shown in the previous section, and thus we tried a different approach to increase activity. Our previously published single-substitution study showed that the replacement of Tyr with Ala at the 97th position of the ST0452 protein provided the highest specific activity of all the single mutants constructed (6). We therefore attempted to construct and characterize mutant ST0452 proteins using site saturation mutagenesis of the 97th residue (Tyr). The 103rd residue (Tyr) of *EcGlmU*, corresponding to the 97th residue (Tyr) of the ST0452 protein, was predicted to contact the acetyl substituent of the GlcNAc-1-P substrate in a cocrystal structure (8). Also as

TABLE 1 GlcNAc-1-P UTase activity of the wild-type and double-mutant ST0452 proteins

Protein	Specific activity ^a ($\mu\text{mol}/\text{min}/\text{mg}$ protein)
ST0452	15.08 \pm 0.34
G9A/T80A	0.43 \pm 0.01
G9A/Y97A	ND
G9A/Y97F	1.04 \pm 0.01
G9A/K147A	0.21 \pm 0.02
T80A/Y97A	0.48 \pm 0.01
T80A/Y97F	0.60 \pm 0.01
T80A/K147A	1.36 \pm 0.02
Y97A/K147A	1.27 \pm 0.04
Y97F/K147A	2.78 \pm 0.08

^aND, nondetectable. All experiments were repeated three times.

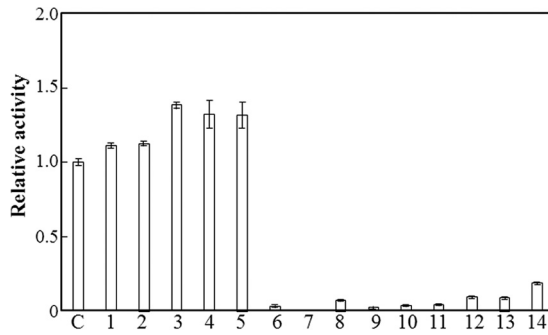


FIG 2 GlcNAc-1-P UTase activity of wild-type, single-mutant, and double-mutant ST0452 proteins. Enzymatic activities were measured for 2 min at 80°C. The relative activity is expressed as the ratio of the activity of the protein of interest to that of the wild-type ST0452 protein. Lane C, the wild-type ST0452 protein; lane 1, G9A; lane 2, T80A; lane 3, Y97A; lane 4, Y97F; lane 5, K147A; lane 6, G9A/T80A; lane 7, G9A/Y97A; lane 8, G9A/Y97F; lane 9, G9A/K147A; lane 10, T80A/Y97A; lane 11, T80A/Y97F; lane 12, T80A/K147A; lane 13, Y97A/K147A; lane 14, Y97F/K147A. All experiments were repeated three times.

shown in our previous publication (6), the Tyr residue is conserved between *EcGlmU* and ST0452 protein; however, this residue is specific to GlmU and ST0452 protein and is not present in RmlA. Therefore, this residue was thought to determine substrate specificity, and thus it was chosen as a target for substitution.

All mutant ST0452 proteins with a Y97 substitution were successfully expressed in *E. coli*. After the cells were harvested and debris was removed by centrifugation, each supernatant was treated at 80°C for 20 min. As shown in Fig. 3, all 19 mutant ST0452 proteins remained in the soluble fraction, revealing that these substitutions did not decrease the thermostability and solubility. In our previous work on single substitutions

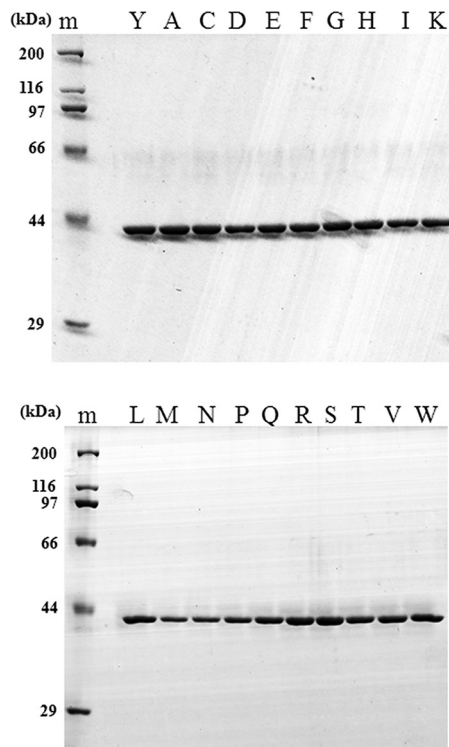


FIG 3 SDS-PAGE analysis of the wild-type and mutant ST0452 proteins. The recombinant proteins, expressed in *E. coli* and treated at 80°C for 20 min, were analyzed on PAGE gels containing 0.1% SDS. Lane m, standard molecular mass proteins; lane Y, the wild-type ST0452 protein. Other lanes are named according to the substitution for Y97; for example, lane A represents the Y97A mutant protein. The proteins were visualized by staining with Coomassie brilliant blue R-250.

TABLE 2 GlcNAc-1-P UTase and Glc-1-P UTase specific activities of the mutant ST0452 proteins

Protein	Specific activity ($\mu\text{mol}/\text{min}/\text{mg protein}$) ^a	
	GlcNAc-1-P UTase	Glc-1-P UTase
ST0452	15.08 \pm 0.34	4.08 \pm 0.04
Y97A	27.52 \pm 0.22	1.74 \pm 0.29
Y97C	10.30 \pm 0.59	2.18 \pm 0.24
Y97D	21.06 \pm 0.74	0.66 \pm 0.19
Y97E	7.04 \pm 0.65	ND
Y97F	18.40 \pm 0.69	3.35 \pm 0.15
Y97G	18.12 \pm 1.32	0.98 \pm 0.14
Y97H	43.70 \pm 3.63	10.41 \pm 0.49
Y97I	18.68 \pm 1.36	ND
Y97K	20.37 \pm 1.07	1.09 \pm 0.07
Y97L	22.03 \pm 0.49	9.97 \pm 0.54
Y97M	19.51 \pm 1.36	11.45 \pm 0.81
Y97N	67.11 \pm 3.38	16.92 \pm 0.58
Y97P	10.31 \pm 0.67	ND
Y97Q	34.60 \pm 1.64	4.26 \pm 0.10
Y97R	ND	ND
Y97S	29.77 \pm 1.70	4.51 \pm 0.37
Y97T	31.27 \pm 0.50	1.03 \pm 0.14
Y97V	53.79 \pm 0.65	1.22 \pm 0.29
Y97W	ND	ND

^aND, nondetectable.

with Ala, we analyzed GlcNAc-1-P UTase and Glc-1-P TTase activities because these activities were typical of GlmU and RmlA, respectively. In this study, we analyzed the direct effect of the sugar-1-P substrate on activity by using the same UTP as a nucleotide substrate for measuring both GlcNAc-1-P UTase and Glc-1-P UTase activities.

The specific activities of the two sugar-1-P NTase activities obtained for each mutant ST0452 protein are shown in Table 2 and indicate that 14 mutant ST0452 proteins exhibit higher GlcNAc-1-P UTase activities than the activity of the wild-type ST0452 protein. Of these, six mutants also exhibited increased Glc-1-P UTase activity. The relative GlcNAc-1-P UTase and Glc-1-P UTase activities are shown in Fig. 4. An increase in both enzymatic activities was observed with six mutant proteins (Y97N, Y97H, Y97Q, Y97S, Y97L, and Y97M), whereas an increase in just GlcNAc-1-P UTase activity was obtained with eight mutant proteins (Y97V, Y97T, Y97A, Y97D, Y97K, Y97I, Y97F, and Y97G). For GlcNAc-1-P UTase activity, the substitution of Ala for Tyr provided 2-times-higher GlcNAc-1-P UTase activity (6), whereas the Y97N mutant exhibited 4-times-higher activity than the wild-type, with activity comparable to that of the *E. coli* enzyme

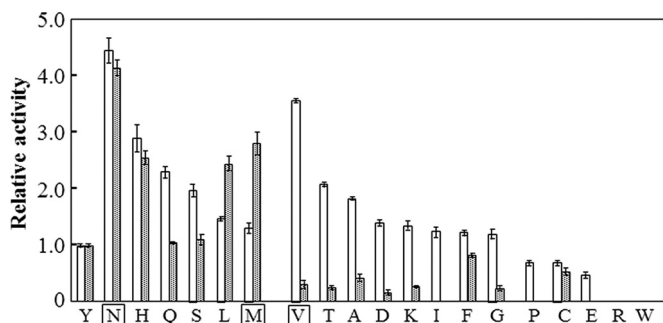


FIG 4 GlcNAc-1-P UTase and Glc-1-P UTase activities of the wild-type and mutant ST0452 proteins. The relative activities of GlcNAc-1-P UTase activity (open bars) and Glc-1-P UTase (hatched bars) are shown. The relative activity is expressed as a ratio of each activity measured for the protein of interest to that of the wild-type ST0452 protein. The symbols used in this figure for the mutant proteins are the same as the symbols used in Fig. 3 (e.g., Y represents the wild-type ST0452 protein, and A represents the Y97A mutant protein). The boxed symbols represent typical mutant proteins used for kinetic analyses. All experiments were repeated three times.

TABLE 3 Kinetic constants for the GlcNAc-1-P and Glc-1-P UTase activities of the wild-type and Y97 mutant ST0452 proteins^a

Protein	GlcNAc-1-P UTase activity			Glc-1-P UTase activity		
	K_m (mM) ^b	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m (mM) ^c	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
ST0452	0.068 ± 0.009	9.97 ± 0.36	146.6	1.23 ± 0.18	2.97 ± 0.11	2.41
Y97N	0.147 ± 0.019	49.75 ± 1.66	338.4	2.53 ± 0.36	38.15 ± 2.03	15.08
Y97M	0.097 ± 0.020	10.90 ± 0.55	112.4	1.74 ± 0.15	14.97 ± 0.46	8.60
Y97V	0.861 ± 0.078	48.95 ± 1.21	56.9	3.28 ± 0.58	2.08 ± 0.16	0.63

^aAssays were carried out in triplicate.

^bThe value was determined for GlcNAc-1-P.

^cThe value was determined for Glc-1-P.

EcGlmU. Although the Y97V mutant protein exhibited no increase in Glc-1-P UTase activity, its GlcNAc-1-P UTase activity was over 3.5 times higher than that of the wild-type ST0452 protein. For the Y97M and Y97L mutants, the rate of increase for Glc-1-P UTase activity (relative to the wild-type value) was higher than the rate of increase for GlcNAc-1-P UTase activity (relative to the wild-type value).

Three typical mutant proteins, Y97N (with the highest GlcNAc-1-P UTase activity of the mutant ST0452 proteins with increases in both GlcNAc-1-P UTase and Glc-1-P UTase activities), Y97M (with a higher ratio of Glc-1-P UTase activity than Y97L), and Y97V (with the highest GlcNAc-1-P UTase activity of the mutant ST0452 proteins with increases only of GlcNAc-1-P UTase activity) were chosen for detailed kinetic analysis (Fig. 4, boxed N, M, and V residues, respectively).

Kinetic constants of Y97M, Y97N, and Y97V. To detect the effect of substrate concentration on the GlcNAc-1-P UTase and Glc-1-P UTase activities of the three mutant proteins, kinetic analyses using the Michaelis-Menten equation were performed. The apparent K_m for each sugar-1-P substrate was determined using 1 mM UTP, a concentration approximately 100 to 500 times higher than its apparent K_m value. For the forward reaction, the direction producing UDP-GlcNAc from GlcNAc-1-P and UTP or UDP-Glc from Glc-1-P and UTP, the apparent K_m for sugar-1-P substrate and apparent k_{cat} values of each mutant protein obtained are shown in Table 3. The apparent k_{cat} values for GlcNAc-1-P UTase and Glc-1-P UTase activities of the Y97N protein were, respectively, 49.75 ± 1.66 and 38.15 ± 2.03 s⁻¹, 5.0 and 12.8 times greater than those of wild-type ST0452 protein. The Y97M protein provided an apparent k_{cat} value for Glc-1-P UTase activity of 14.97 ± 0.46 s⁻¹, 5.0 times greater than that of the wild-type protein. The Y97V protein showed an apparent k_{cat} value for GlcNAc-1-P UTase activity of 48.95 ± 1.21 s⁻¹, 4.9 times higher than that of the wild-type ST0452 protein, but an apparent k_{cat} value for Glc-1-P UTase activity of 2.08 ± 0.16 s⁻¹, representing a 30% decrease in the value for the wild-type ST0452 protein. These results indicate that the increased activity exhibited by typical mutant ST0452 proteins was mainly due to an increase in the apparent k_{cat} value.

Evaluation of the effect of substitution to increase activity by introducing the same mutation into the EcGlmU protein. The results obtained from replacing the 97th residue (Tyr) in the ST0452 protein with the other 19 amino acids showed that substitution by any one of 14 amino acids caused an increase in GlcNAc-1-P UTase activity, mainly due to an increase in their k_{cat} values. The 97th residue of the ST0452 protein was deemed an important residue for interaction with the acetyl substituent in the reaction center based on three-dimensional structures obtained from cocrystals of the bacterial GlmU and substrate (8, 9, 10). Amino acid residues playing an important role in the reaction center are conserved between the archaeal ST0452 protein and similar bacterial enzymes, suggesting that enhanced activity might be obtained by introducing similar substitutions into the bacterial enzymes.

The replacement of an important residue in the reaction center with Ala usually has a different type of effect on enzymatic activity. In the ST0452 protein, changing the 97th residue from Tyr to Ala actually increased GlcNAc-1-P UTase activity (6). We decided to evaluate whether the same effect would be observed with the *E. coli* GlmU protein. The 103rd residue (Tyr) corresponds to the 97th residue in the ST0452 protein,

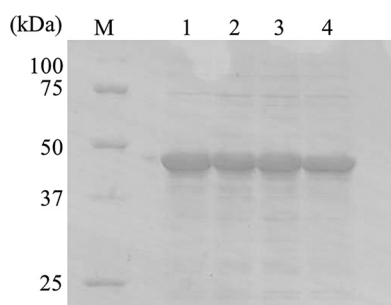


FIG 5 SDS-PAGE analysis of the wild-type and mutant *EcGlmU* proteins. The recombinant proteins, expressed in *E. coli* and purified with a His tag, were analyzed on PAGE gels containing 0.1% SDS. Lane M, standard molecular mass proteins; lane 1, wild-type *EcGlmU* protein; lane 2, Y103A; lane 3, Y103N; lane 4, Y103V. The proteins were visualized by staining with Coomassie brilliant blue R-250.

and thus the mutant Y103A *EcGlmU* was constructed and compared to the wild-type *EcGlmU* enzyme. The mutant and wild-type recombinant proteins were easily expressed in *E. coli* (Fig. 5). *EcGlmU* can catalyze GlcNAc-1-P but not Glc-1-P, and therefore only GlcNAc-1-P UTase activities were measured. As shown in Table 4, the specific activity of the wild-type *EcGlmU* protein was 5 times higher than that of the wild-type ST0452 protein. The Y103A mutant protein showed a 20% increase in activity compared to that of the wild-type *EcGlmU*, consistent with the finding by Wang et al. (11). Consequently, we attempted to construct mutant *EcGlmU* proteins in which the Tyr at the 103rd position was replaced by Asn or Val because the former substitution in the ST0452 protein provided the highest GlcNAc-1-P activity among mutants exhibiting increases in both GlcNAc-1-P UTase and Glc-1-P UTase activities, whereas the latter provided the highest GlcNAc-1-P UTase activity among mutants exhibiting an increase only in the GlcNAc-1-P UTase activity.

Expression vectors were constructed encoding *EcGlmU* mutants with substitution of the 103rd residue by Asn or Val. The recombinant proteins were easily expressed in *E. coli* and purified (Fig. 5), and their specific activities are summarized in Table 4. The introduction of Val at the 103rd position of *EcGlmU* resulted in activity 30% lower than that of the wild-type *EcGlmU*. However, substitution with Asn resulted in 1.3-times-higher GlcNAc-1-P UTase activity than that of the wild-type *EcGlmU* protein. Although this increase is smaller than that observed in the mutant ST0452 protein with the same substitution (a 4.45 times increase), the results support the same trend for this substitution in both enzymes.

DISCUSSION

In the current study, our primary aim was to generate mutant ST0452 proteins exhibiting highly enhanced enzymatic activities based on the premise that combina-

TABLE 4 Effects of Tyr97 mutation of ST0452 protein from *S. tokodaii* and Tyr103 mutation of *GlmU* from *E. coli* strain K-12 on GlcNAc-1-P UTase activities^a

Protein	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	Relative activity (%)
ST0452		
Wild type	15.08 \pm 0.34	100.0
Y97A	27.52 \pm 0.22	182.5
Y97N	67.11 \pm 3.39	445.0
Y97V	53.79 \pm 0.65	356.7
<i>EcGlmU</i>		
Wild type	75.63 \pm 4.11	100.0
Y103A	93.97 \pm 1.96	124.2
Y103N	99.80 \pm 7.38	132.0
Y103V	52.65 \pm 2.82	69.6

^aAll experiments were repeated three times.

tions of two mutations would significantly increase activity. However, all the double-mutant ST0452 proteins exhibited only trace levels of activity. Double substitutions in which Thr, Tyr, or Lys was replaced by Ala could have reduced the polarity or electrical charge in the reaction center, whereas a single substitution might optimize the interaction between the ST0452 protein and substrate or product. However, two substitutions with Ala in the reaction center may cause a weak interaction between the enzyme and the substrate or product. The reasons underlying this loss of activity require more detailed analyses, including determination of the three-dimensional structure of a cocrystal of a double-mutant ST0452 protein and GlcNAc-1-P substrate.

Fourteen substitution mutants exhibited higher GlcNAc-1-P UTase activity than the wild-type ST0452 protein, and two, Y97N and Y97H, showed similar increases in both GlcNAc-1-P UTase and Glc-1-P UTase activities. Glutamine, the third effective amino acid, contains a nitrogen atom in the side chain, as do asparagine and histidine, and this nitrogen-containing residue might play a role in catalysis. A comparison of the activities of wild-type ST0452 protein and Y97F indicates that the OH group is not very important for catalysis. Two mutant proteins, Y97R and Y97W, exhibited no activity, suggesting that amino acid residues larger than Tyr might inhibit activity. It remained unclear what specific and common features of various mutations cause an increase in both GlcNAc-1-P UTase and Glc-1-P UTase activities, an increase in only GlcNAc-1-P UTase activity, or a decrease in both activities. Substitution with Asn was the most effective mutation for increasing both activities, and Asn is a polar amino acid. However, Val is nonpolar, and it was the second most effective amino acid for increasing GlcNAc-1-P UTase activity. The mechanism underlying increased sugar-1-P NTase activity by substitution of the 97th residue of the ST0452 proteins remains unclear, and more detailed analyses, including determination of the three-dimensional structures of these mutant proteins, are required.

The GlcNAc-1-P UTase activity of the ST0452 protein was increased by changing the 97th residue from Tyr to any one of 14 other amino acids. The GlcNAc-1-P UTase activity of *EcGlmU* was unaffected or increased 1.5 times by changing the corresponding 103rd residue from Tyr to Ala or Phe, as shown in Table 4, consistent with the results reported by Wang et al. (11) and revealing that the activities of both mesophilic enzymes and thermostable enzymes from archaea can be increased by substitution.

Substitution with amino acid residues smaller than the original 97th (103rd in *EcGlmU*) Tyr residue of the ST0452 protein increased the GlcNAc-1-P UTase activities of both ST0452 and *EcGlmU* proteins. Similarly, in the bacterial sugar-1-P TTase enzyme, substitution with smaller amino acid residues expands the nucleoside triphosphate or sugar-1-P substrate specificity (12, 13, 14, 15). Structure-based substitution mutants have been designed for expansion of the substrate specificity, primarily due to improvement in K_m values (13). A combination of random mutagenesis and site-saturated mutagenesis methods has succeeded in creating mutant bacterial RmlA with increased specific activity of the sugar-1-P NTase activity, indicating that conversion of Gln residues at three different positions to smaller amino acids increases specific activity of the sugar-1-P NTase activity, and analyses of typical mutant clones indicated that their activity increase is mainly due to increases of apparent k_{cat} values (14). Combining these observations and our current results, it can be concluded that conversion to smaller amino acids in the reaction center increases sugar-1-P NTase activity.

Kinetic analyses of three typical mutant ST0452 proteins indicate that this activity increase is mainly due to an increase in k_{cat} values. However, most mutant ST0452 proteins with increased GlcNAc-1-P UTase activity also showed increases in K_m values. From these observations, it could be hypothesized that mutation at the 97th position (Tyr) might weaken the interaction between the reaction center of the ST0452 protein and substrate; thus the release rate of product might be greater than that of the wild type. To confirm this hypothesis, more detailed analyses are required.

The target ST0452 protein is a bifunctional enzyme exhibiting sugar-1-P NTase and

TABLE 5 Primers used to construct the double-mutant ST0452 proteins

Primer name	Sequence ^a
dT80AF	TAAAAGGGG CGGGT GCGGCCATATTGTCTG
dT80AR	GCCGCACCC CGCCCT TTTTATATCATCTTTC
dY97AF	CACTATAAAT GCGGG GAGATTATTCTTTTC
dY97AR	TAAATCTCC CGCA ATTATAAGTGCTTCATCG
dY97FF	CTTATAAAT TTTGG GAGATTATTCTTTCAAAC
dY97FR	AATCTCC AAA AATTATAAGTGCTTCATCG
dK147AF	ATTATAGA AGGCC AGAGATACCTCCATC
dK147AR	GTATCTCT GCGCT TCTATAATTTAGATAAG

^aUnderlining indicates the overlap regions introduced into each primer. Boldface indicates the substituted nucleotides.

amino-sugar-1-P AcTase activities. Amino-sugar-1-P AcTase activity is encoded at the C terminus of the protein, and this activity is significantly increased by C-terminal truncation (16). To enhance the utility of this bifunctional enzyme for the production of sugar nucleotides, the present Y97N mutant should be combined with a mutation that increases amino-sugar-1-P AcTase activity, resulting in a mutant ST0452 protein exhibiting two high activities. Mutant ST0452 proteins exhibiting increased activities could be used for the synthesis of useful materials and for fundamental analyses of mechanisms for increasing enzymatic activity.

MATERIALS AND METHODS

General materials and microorganisms. UTP, GlcNAc-1-P, Glc-1-P, UDP-GlcNAc, and UDP-Glc were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The restriction enzymes NdeI, XhoI, and DpnI and a DNA ligation kit (Mighty Mix) were obtained from TaKaRa Bio Inc. (Shiga, Japan). KOD-plus-Neo DNA polymerase was purchased from Toyobo Co., Ltd. (Osaka, Japan). *E. coli* strains JM109 for DNA manipulation and BL21-CodonPlus(DE3)-RIPL for protein expression were obtained, respectively, from TaKaRa Bio, Inc., and Stratagene (La Jolla, CA, USA). The plasmid vector pET-21a(+) was purchased from Novagen (Madison, WI, USA). A His SpinTrap Talon column and Amicon Ultra 0.5-ml Ultracel-30K filter (unit with a nominal molecular weight limit of 30,000) were obtained, respectively, from GE Healthcare Life Sciences Corp. (Pittsburgh, PA, USA) and Merck Millipore, Ltd. (Darmstadt, Germany).

Primers used and primer combinations. The sequences of all primers designed for construction of the double mutant and the combination of primers and template plasmids used are shown in Tables 5 and 6, respectively. The nucleotide sequences of all primers designed for site saturation mutagenesis of the 97th residue (Tyr) in the ST0452 protein and the combination of primers used for PCR amplification are shown in Tables 7 and 8, respectively. The sequences of primers for PCR amplification of the *E. coli* GImU gene and the mutated *E. coli* GImU gene, together with the combination of primers used are shown in Tables 9 and 10, respectively.

Construction of expression vectors. ST0452 expression vectors with double substitution mutations were constructed using appropriate combinations of the previously constructed mutant ST0452 expression vectors and primer sets shown in Table 6. PCRs to introduce the second substitution were performed in 50 μ l of standard PCR mixture with 50 ng of template plasmid DNA and 15 pmol each of the appropriate set of primers using the following temperature cycle: 2 min at 94°C, followed by 13 cycles of 98°C for 10 s, 50°C for 30 s, and 68°C for 6 min 40 s. The amplified PCR fragments were digested with the restriction enzyme DpnI at 37°C for 1 h, and then the digested DNA was directly introduced into *E. coli* strain JM109. After confirmation by sequencing, each constructed plasmid was designated by the name shown in Table 6.

Mutant ST0452 expression vectors with substitution of the 97th residue (Tyr) by any of the other 19 L-amino acids were constructed using PCR amplification with the primer sets shown in Table 8 and

TABLE 6 List of primer sets and template plasmids used to construct the double-mutant ST0452 genes with names of the product plasmids and proteins

Forward primer	Reverse primer	Template plasmid	Product plasmid	Protein name
dT80AF	dT80AR	pST0452(G9A)H	pST0452(G9A)(T80A)H	G9A/T80A
dY97AF	dY97AR	pST0452(G9A)H	pST0452(G9A)(Y97A)H	G9A/Y97A
dY97FF	dY97FR	pST0452(G9A)H	pST0452(G9A)(Y97F)H	G9A/Y97F
dK147AF	dK147AR	pST0452(G9A)H	pST0452(G9A)(K147A)H	G9A/K147A
dY97AF	dY97AR	pST0452(T80A)H	pST0452(T80A)(Y97A)H	T80A/Y97A
dY97FF	dY97FR	pST0452(T80A)H	pST0452(T80A)(Y97F)H	T80A/Y97F
dK147AF	dK147AR	pST0452(T80A)H	pST0452(T80A)(K147A)H	T80A/K147A
dK147AF	dK147AR	pST0452(Y97A)H	pST0452(Y97A)(K147A)H	Y97A/K147A
dK147AF	dK147AR	pST0452(Y97F)H	pST0452(Y97F)(K147A)H	Y97F/K147A

TABLE 7 Primers used for substitution of the Tyr at the 97th position by other amino acids

Primer name	Sequence ^a
dY97CF	CACTTATAA TTGCGG GAGATTATTCTTTTC
dY97CR	TAAATCTCC GCA AATTATAAGTGCTTCATCG
dY97DF	CACTTATAA TGAT GGAGATTATTCTTTTC
dY97DR	ATCTCC ATC AATTATAAGTGCTTCATCG
dY97EF	CACTTATAA TGA GGAGATTATTCTTTTC
dY97ER	ATCTCC TC AATTATAAGTGCTTCATCG
dY97GF	CACTTATAA TGGCGG GAGATTATTCTTTTC
dY97GR	TAAATCTCC GCC AATTATAAGTGCTTCATCG
dY97HF	CACTTATAA TCAT GGAGATTATTCTTTTC
dY97HR	ATCTCC ATG AATTATAAGTGCTTCATCG
dY97IF	CACTTATAA TATT GGAGATTATTCTTTTC
dY97IR	ATCTCC CA AATTATAAGTGCTTCATCG
dY97KF	CACTTATAA TAA GGAGATTATTCTTTTC
dY97KR	TAAATCTCC TTT AATTATAAGTGCTTCATCG
dY97LF	CTTATAA TTCTG GGAGATTATTCTTTTC
dY97LR	ATCTCC CA GAAATTATAAGTGCTTCATCG
dY97MF	CACTTATAA TATG GGAGATTATTCTTTTC
dY97MR	TAAATCTCC CAT AATTATAAGTGCTTCATCG
dY97NF	CACTTATAA TAA TGGAGATTATTCTTTTC
dY97NR	TAAATCTCC ATA AATTATAAGTGCTTCATCG
dY97PF	CACTTATAA TCGG GGAGATTATTCTTTTC
dY97PR	ATCTCC CGG AATTATAAGTGCTTCATCG
dY97QF	CACTTATAA TCAG GGAGATTATTCTTTTC
dY97QR	ATCTCC CTG AATTATAAGTGCTTCATCG
dY97RF	CACTTATAA TCG GGAGATTATTCTTTTC
dY97RR	TAAATCTCC GCG AATTATAAGTGCTTCATCG
dY97SF	CTTATAA TTAG GGAGATTATTCTTTTC
dY97SR	ATCTCC GCT AATTATAAGTGCTTCATCG
dY97TF	CACTTATAA TACG GGAGATTATTCTTTTC
dY97TR	ATCTCC GGT AATTATAAGTGCTTCATCG
dY97VF	CACTTATAA TGTG GGAGATTATTCTTTTC
dY97VR	ATCTCC CA AATTATAAGTGCTTCATCG
dY97WF	CACTTATAA TTGG GGAGATTATTCTTTTC
dY97WR	TAAATCTCC CCA AATTATAAGTGCTTCATCG

^aUnderlining indicates the overlap regions introduced into each primer. Boldface indicates the substituted codons.

pETST0452H plasmid DNA as the template. The cycle profiles for these PCRs were as given above. The amplified PCR fragments were digested with the restriction enzyme DpnI at 37°C for 1 h, and then the digested DNA was directly introduced into *E. coli* strain JM109. After confirmation by sequencing, each constructed plasmid was designated as shown in Table 8.

TABLE 8 List of primer sets, template plasmids used for construction of substitution mutant ST0452 genes, plasmids used as templates, and names of the product plasmids and proteins

Forward primer	Reverse primer	Template plasmid	Plasmid name	Protein name
dY97CF	dY97CR	pST0452H	pST0452(Y97C)H	Y97C
dY97DF	dY97DR	pST0452H	pST0452(Y97D)H	Y97D
dY97EF	dY97ER	pST0452H	pST0452(Y97E)H	Y97E
dY97GF	dY97GR	pST0452H	pST0452(Y97G)H	Y97G
dY97HF	dY97HR	pST0452H	pST0452(Y97H)H	Y97H
dY97IF	dY97IR	pST0452H	pST0452(Y97I)H	Y97I
dY97KF	dY97KR	pST0452H	pST0452(Y97K)H	Y97K
dY97LF	dY97LR	pST0452H	pST0452(Y97L)H	Y97L
dY97MF	dY97MR	pST0452H	pST0452(Y97M)H	Y97M
dY97NF	dY97NR	pST0452H	pST0452(Y97N)H	Y97N
dY97PF	dY97PR	pST0452H	pST0452(Y97P)H	Y97P
dY97QF	dY97QR	pST0452H	pST0452(Y97Q)H	Y97Q
dY97RF	dY97RR	pST0452H	pST0452(Y97R)H	Y97R
dY97SF	dY97SR	pST0452H	pST0452(Y97S)H	Y97S
dY97TF	dY97TR	pST0452H	pST0452(Y97T)H	Y97T
dY97VF	dY97VR	pST0452H	pST0452(Y97V)H	Y97V
dY97WF	dY97WR	pST0452H	pST0452(Y97W)H	Y97W

TABLE 9 Primers used to construct the expression vector for wild-type and mutant *EcGlmU* proteins

Primer name	Sequence ^a
dEcGlmUF	ACTGGcatatgTTGAATAATGCTATGAGCG
dEcGlmUR	TCAAActcgagCTTTTTCTTTACCGGACGAC
dEcGlmUY103AF	TGCTC CG GGGCGACGTGCCGCTGATCTCTG
dEcGlmUY103AR	GTCGCC CG GAGCATTAAAATGTCTTCATC
dEcGlmUY103NF	TGCTCA AT GGGCGACGTGCCGCTGATCTCTG
dEcGlmUY103NR	GTCGCC ATT GAGCATTAAAATGTCTTCATC
dEcGlmUY103VF	TGCTC GT GGGCGACGTGCCGCTGATCTCTG
dEcGlmUY103VR	GTCGCC CAC GAGCATTAAAATGTCTTCATC

^aLowercase letters indicate the NdeI site, and italicized lowercase letters indicate the XhoI site. Underlining indicates the overlap regions introduced into each primer. Boldface indicates substitutions.

The *EcGlmU* protein exhibits GlcNAc-1-P UTase activity similar to that of the ST0452 protein, and PCR amplification of this gene was performed with *E. coli* genomic DNA and the primers dEcGlmUF and dEcGlmUR, as shown in Tables 9 and 10. The amplified fragment was digested with NdeI and XhoI, and the digested fragment was ligated with pET-21a(+) DNA digested with the same restriction enzymes. The constructed expression vector for the *EcGlmU* protein with a His tag at its C terminus was confirmed by sequencing and designated pEcGlmUH. The expression vector for the mutant *EcGlmU* gene, encoding the 103rd residue (Tyr) replaced by Ala, Asn, or Val, was constructed by PCR amplification using the primer sets shown in Table 10 and the parental expression vector, pEcGlmUH. The amplified fragments were digested with DpnI, and the digested fragments were directly introduced into *E. coli* strain JM109. The substitutions were confirmed by sequencing, and the constructed plasmid vectors were designated as shown in Table 10.

Expression and purification of the recombinant proteins. The constructed expression vectors were introduced into *E. coli* strain BL21-CodonPlus(DE3)-RIPL cells, and then the cells were grown in 5 ml of Luria-Bertani (LB) medium containing 100 μ g/ml ampicillin and 50 μ g/ml chloramphenicol at 37°C to an absorbance at 600 nm (A_{600}) of 0.6. Isopropyl β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and aeration was continued for 3.5 h at 37°C to induce the expression of recombinant protein. The cells were then harvested by centrifugation at 20,000 \times *g* for 2 min at 4°C, and the pelleted cells were suspended in 200 μ l of 50 mM Tris-HCl (pH 7.5). The suspended cells were ruptured by sonication with a Bioruptor UCD-200TM (Cosmo Bio Co., Ltd., Tokyo, Japan) using 10 cycles of 30-s pulses followed by a 30-s rest on ice. The suspension was centrifuged at 20,000 \times *g* for 10 min at 4°C, and the supernatant was collected as the soluble fraction. For all proteins except recombinant wild-type and mutant *EcGlmU* proteins, the soluble fraction was treated at 80°C for 20 min and then centrifuged at 20,000 \times *g* for 10 min at 4°C. Recombinant proteins with a His tag at the C terminus were purified using a His SpinTrap Talon column according to the manufacturer's instructions. The buffer solution containing the recombinant protein was changed to 50 mM Tris-HCl (pH 7.5) using an Amicon Ultra 0.5-ml Ultracel 30K filter. Purified recombinant protein was stored at 4°C. Purified protein was separated by 0.1% SDS–12% polyacrylamide gel electrophoresis (SDS-PAGE) and detected with Coomassie brilliant blue R-250. The concentration of each purified protein was determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA, USA).

Enzymatic assays. The sugar-1-P UTase activities of the recombinant proteins were analyzed essentially as described previously (4), with some modifications. The reactions for thermostable recombinant proteins were performed in a 20- μ l reaction solution containing 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM UTP, 10 mM sugar-1-P, and 10 ng of purified recombinant protein. After 2 min of preincubation of the reaction mixture without protein at 80°C, the reaction was started by the addition of the recombinant protein and progressed at 80°C (2 min for GlcNAc-1-P UTase and 5 min for Glc-1-P UTase activities) for thermostable recombinant proteins. The mesophilic proteins from *E. coli* were assayed in the same manner, but all steps were conducted at 37°C. Following incubation, the reaction mixtures were immediately cooled on ice to terminate the reaction and then mixed with 180 μ l of 500 mM KH₂PO₄ to adjust the solution content to that used in high-performance liquid chromatography (HPLC) separation. For reactions producing UDP-Glc, an additional 200 μ l of 80 mM triethylamine-phosphate buffer (pH 6.5) was added to adjust the solution content to that used in HPLC separation. For reactions catalyzed by *E. coli* proteins, 5 μ l of acetate was added before the addition of 175 μ l of 500 mM KH₂PO₄ to terminate the reaction.

TABLE 10 List of primer sets, templates used to construct the wild-type and mutant *EcGlmU* genes, and names of the product plasmids and proteins

Forward primer	Reverse primer	Template	Plasmid name	Protein name
dEcGlmUF	dEcGlmUR	<i>E. coli</i> genomic DNA	pEcGlmUH	<i>EcGlmU</i>
dEcGlmUY103AF	dEcGlmUY103AR	pEcGlmUH	pEcGlmUY103AH	Y103A
dEcGlmUY103NF	dEcGlmUY103NR	pEcGlmUH	pEcGlmUY103NH	Y103N
dEcGlmUY103VF	dEcGlmUY103VR	pEcGlmUH	pEcGlmUY103VH	Y103V

The amount of sugar nucleotide produced was determined by analyzing a 50- μ l aliquot of the solution on a Waters Alliance HPLC e2695 system (Waters, Milford, MA, USA) equipped with a Wakosil 5C18-200 column (0.46 by 25 cm; Wako, Osaka, Japan). For analyses of UDP-GlcNAc, the flow rate of 500 mM KH_2PO_4 was maintained at 0.8 ml/min, and the column temperature was maintained at 60°C. To measure UDP-Glc, the flow rate of 40 mM triethylamine-phosphate buffer (pH 6.5) was maintained at 0.8 ml/min, and the column temperature was maintained at 60°C. The products, UDP-GlcNAc and UDP-Glc, were monitored by absorbance at 254 nm. The amount of product was calculated from the area under the peaks. The retention times of UTP and UDP-GlcNAc under conditions using the KH_2PO_4 solution described above were 3.7 and 4.1 min, respectively, and those of UTP and UDP-Glc under conditions using the trimethylamine-phosphate buffer were 10 and 6 min, respectively.

Pseudo-first-order kinetics were obtained by fixing UTP at a saturating concentration of 1 mM, a concentration approximately 100 to 500 times higher than its apparent K_m value, and titrating sugar-1-P. Different concentrations in the range from 0.01 to 10 mM for GlcNAc-1-P and from 0.5 to 7 mM for Glc-1-P were assayed in triplicate. Reaction rates were confirmed to be linear over twice the incubation time and were corrected for time and enzyme concentrations. The kinetic curves were generated to fit to the Michaelis-Menten equation $\{\text{rate} = V_{\text{max}} \times [S]/(K_m + [S])\}$ using SigmaPlot, version 12.5 (Systat Software, San Jose, CA).

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