Crystal structure of Vibrionaceae Photobacterium sp. JT-ISH-224 α2,6-sialyltransferase in a ternary complex with donor product CMP and acceptor substrate lactose: catalytic mechanism and substrate recognition

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Sialyltransferases are a family of glycosyltransferases that catalyze the transfer of N-acetylneuraminic acid residues from cytidine monophosphate N-acetylneuraminic acid (CMP-NeuAc) as a donor substrate to the carbohydrate groups of glycoproteins and glycolipids as acceptor substrates. We determined the crystal structure of A16psp26ST, the N-terminal truncated form of α2,6-sialyltransferase from Vibrionaceae Photobacterium sp. JT-ISH-224, complexed with a donor product CMP and an acceptor substrate lactose. A16psp26ST has three structural domains. Domain 1 belongs to the immunoglobulin-like beta-sandwich fold, and domains 2 and 3 form the glycosyltransferase-B structure. The CMP and lactose were bound in the deep cleft between domains 2 and 3. In the structure, only Asp232 was within hydrogen-binding distance of the acceptor O6 carbon of the galactose residue in lactose, and His405 was within hydrogen-binding distance of the phosphate oxygen of CMP. Mutation of these residues greatly decreased the activity of the enzyme. These structural and mutational results indicated that Asp232 might act as a catalytic base for deprotonation of the acceptor substrate, and His405 might act as a catalytic acid for protonation of the donor substrate. These findings are consistent with an in-line-displacement reaction mechanism in which A16psp26ST catalyzes the inverting transfer reaction. Unlike the case with multifunctional sialyltransferase (∆24PmST1) complexed with CMP and lactose, the crystal structure of which was recently reported, the α2,6 reaction specificity of A16psp26ST is likely to be determined by His123.

Keywords: α2,6-sialyltransferase/crystal structure/substrate assisted catalytic mechanism/Vibrionaceae Photobacterium sp. JT-ISH-224

Introduction

Sialic acids often exist in the carbohydrate moiety of mammalian glycoconjugates, such as glycoproteins and glycolipids, and are linked to the terminal positions of the carbohydrate chains of the glycoconjugates. Many reports have demonstrated that the sialylated carbohydrate chains play important roles in a number of biological events, such as immunological response, cell–cell recognition, and inflammation (Varki 1993; Gagneux and Varki 1999). Four main linkage patterns—NeuAcα2-6Gal, NeuAcα2-3Gal, NeuAcα2-6GalNAc, and NeuAcα2-8NeuAc—are found in the glycoconjugates, and these linkage patterns are formed by specific sialyltransferases. Sialic acid is a component of lipooligosaccharides in pathogenic bacteria such as Campylobacter jejuni, Neisseria gonorrhoeae, and Neisseria meningitidis. Moreover, research on sialyltransferases from diverse bacteria has recently become popular, and many bacterial sialyltransferases have now been reported. To date, bacterial sialyltransferases have been reported from the genera Neisseria (Edwards et al. 1994; Gilbert et al. 1996), Escherichia (Weisgerber et al. 1991), Campylobacter (Gilbert et al. 2000), Haemophilus (Bouz et al. 1999; Hood et al. 2001; Fox et al. 2006), Pasteurella (Yu et al. 2005), Photobacterium (Yamamoto et al. 1996; 1998), Photobacterium phosphoreum (Tsukamoto et al. 2007), Photobacterium leiognathi (Yamamoto et al. 2007), Photobacterium sp. (Okino et al. 2007) and Vibrio sp. (Takakura et al. 2007).

All the sialyltransferases have been classified into five families in the CAZY (carbohydrate-active enzymes) database (Coutinho et al. 2003), i.e., glycosyltransferase family 29, various sialyltransferases from the Eukaryote and viruses; family 38, polysialyltransferase from bacteria such as Escherichia coli and N. meningitidis; family 42, lipooligosaccharide α2,3-sialyltransferase and α2,3/α2,8-sialyltransferase from bacteria such as C. jejuni and Haemophilus influenzae; family 52, α2,3-sialyltransferase from bacteria such as H. influenzae, N. gonorrhoeae, and N. meningitidis; and family 80, α2,6-sialyltransferase and α2,3/α2,6-sialyltransferase from bacteria such as Photobacterium damselae and Pasteurella multocida. Thus four of these families consist of bacterial sialyltransferases.

Up to present, sialyltransferases with three different crystal structures have been reported. The first is a bifunctional enzyme, α2,3/α2,8-sialyltransferase (CstII) from C. jejuni OH4384 in complex with a substrate analog (Chiu et al. 2004). The second is a multifunctional enzyme, α2,3-sialyltransferase (Δ24PmST1) from P. multocida strain P-1059 in the absence and presence of cytidine monophosphate (CMP) (Ni et al. 2006). This enzyme shows α2,3-sialyltransferase activity, α2,6-sialyltransferase activity, sialidase activity, and trans-sialidase activity. Very recently, the crystal structure of this enzyme in complexes with
Our analysis revealed the crystal structure of overall structure. Results and discussion

anism in the sialyltransferases. The first insight into the approximately 40 from C. jejuni we isolated that produced sialyltransferases were classified in bacteria that produce sialyltransferase. Many of the bacteria al. 2007). The third, very recently reported, sia-

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Fig. 1. Overall structure of the Δ16pspST6 complex with CMP, lactose. Secondary structural elements are indicated by ribbons for α-helices, arrows for β-strands. CMP and lactose at active sites are shown as space-fill models with atomic color scheme (C, white; N, blue; O, red; P, orange). All figures were prepared by using PyMOL (DeLano 2002).

acceptor substrate and donor substrate analogs, as well as its substrate-binding sites and catalytic mechanism, has been reported (Ni et al. 2007). The third, very recently reported, sialyltransferase is a monofunctional α2,3-sialyltransferase (CstI) from C. jejuni apo and substrate-analog-bound forms (Chiu et al. 2007).

During the course of previous study, we clarified over 20 bacteria that produce sialyltransferase. Many of the bacteria we isolated that produced sialyltransferases were classified in the genus Photobacterium or the closely related genus Vibrio (Yamamoto et al. 2006). Photobacterium sp. JT-ISH-224 is a bacterium that produce β-galactoside α2,6-sialyltransferase, and we have cloned the gene encoding the sialyltransferase from the bacterium (Okino et al. 2007). This enzyme is thought to be classified into Glycosyltransferase family 80 in the CAZy database and to be related to the formation of glycoconjugate in the bacterium.

In this paper, we report on the crystal structure of the monofunctional β-galactoside α2,6-sialyltransferase Δ16psp26ST, cloned from Photobacterium sp. JT-ISH-224. This provides the first insight into the α2,6 recognition and linkage reaction mechanism in the sialyltransferases.

Results and discussion

Overall structure

Our analysis revealed the crystal structure of Δ16pspST6 (size: approximately 40 × 65 × 95 Å3) (Figure 1). Δ16pspST6 consisted of three structural domains, domains 1 (22–114), 2 (115–338), and 3 (339–514). Domain 1 belongs to the immunoglobulin-like beta-sandwich fold, and domains 2 and 3 form the glycosyltransferase-B (GT-B) structure. The immunoglobulin-like beta-sandwich fold is found in some members of the glycosyl hydrolase family as a noncatalytic domain (Jain et al. 1996). The specific activity of the truncated α2,6-sialyltransferase Δ109pspST6 is two times higher than that of Δ16pspST6 (Table 1). So, domain 1 in Δ16pspST6 may play a role in regulation of the enzymatic activity or locating the matured α2,6-sialyltransferase in the cell. The GT-B structure formed by domains 2 and 3 resembles that of Δ24PmST1. Both the donor substrate product CMP and the acceptor substrate lactose were bound in the deep cleft between domains 2 and 3. Residues involved in CMP binding were isolated to domain 3 of Δ16pspST6, but residues involved in lactose binding were located in both domains 2 and 3. Glycerol was bound between CMP and lactose, and hydrogen bonded to phosphate of CMP, so the glycerol-binding site was predicted to be part of the sialic acid-binding site.

CMP binding

We determined the active site structures of the ternary complex of Δ16pspST6 (Figure 2A). CMP was located to a deep cleft between domains 2 and 3 (Figure 2A and B). However, all residues that interacted with CMP were in domain 3. The side-chain nitrogen of Lys405 was within hydrogen-bonding distances of atoms N-3 and O2 of the cytidine ring. The main chain carbonyls of Lys405 and Gly361 and the main chain nitrogen of His405 were within hydrogen-bonding distance of atom O2 of the cytidine ring. The main chain nitrogen Phe431 was within hydrogen-bonding distance of the oxygen atom O3 of the oxygene atom O2 of the ribose. The side chain of Ser430 was within hydrogen-bonding distance of the oxygen atom O2 of the ribose. The side chains of Ser449 (2.8 Å), His405 (3.2 Å), and water2 (2.6 Å) were within hydrogen-bonding distance of the oxygen atom O3P of the 5′-phosphate. Backbone amide nitrogen and side-chain oxygen from Ser450 (2.7 Å and 2.4 Å) and water17 (2.8 Å) were all within hydrogen-bonding distance to the oxygen atom O2P of the 5′-phosphate.

Lactose binding

Residues involved in acceptor substrate binding were almost entirely isolated to domain 2 of the Δ16pspST6 molecule. The side chain of Asp232 was within hydrogen-bonding distance of the O6 oxygen of the terminal Gal of the lactose. The other oxygen of side chain Asp232 was within hydrogen-bonding distance of O4 of the Gal of lactose. The Trp365 was in hydrophobic contact with the central part of the lactose. The side chain of His204 was within hydrogen-bonding distance of O4 and O3 of the Gal of lactose. The side chain of His123 was within hydrogen-bonding distance of O3 and O5 of the Gal of lactose. The side chain of His123 was within hydrogen-bonding distance of O3 and O5 of the Gal of lactose. The side chain of Gly174 was within hydrogen-bonding distance of O1 of the Glc of lactose. The side chain of Arg153 was within hydrogen-bonding distance of O2 of the Glc of lactose.

Table 1. Comparison of the specific activity of purified Δ16pspST6 and Δ109pspST6

<table>
<thead>
<tr>
<th>Name of enzyme</th>
<th>Specific activity (U/mg of protein)</th>
</tr>
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<tbody>
<tr>
<td>Δ16pspST6</td>
<td>113</td>
</tr>
<tr>
<td>Δ109pspST6</td>
<td>264</td>
</tr>
</tbody>
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...
Acceptors substrate specificity

The binding manner of lactose outlined above suggests that the acceptor-binding site is filled with lactose, and the reducing end of the lactose locates outside of the $\Delta 16pspST6$ molecule. The third sugar moiety of the longer sugar chain would be expected to be outside the substrate-binding cavity. Therefore, $\Delta 16pspST6$ can take the longer sugar chain as a substrate. For example, $\Delta 16pspST6$ can transfer NeuAc from CMP-NeuAc to asiao-fetuin and several pyridylaminated sugar chains (data not shown). In the case of N-acetyllactosamine binding, Arg153 may interact with nitrogen atoms in the N-acetyl residue. The three-dimensional orientation of residues Asp232, His204, His123, Trp365, Asn174, and Arg153 in the substrate-binding cavity may dictate the specificity for the acceptor substrate lactose through interactions with the O6, O5, O4, and O3 hydroxyl groups of Gal and O1 and the O2 hydroxyl groups of Glc.

Glycerol binding

The three glycerol molecules were bound within the crystal structure. One was located between CMP and lactose. Examination of electron density maps at a high contour level ($\sigma$ level = 3) revealed clear electron density at the active site (Figure 2A). The glycerol oxygen bound to Asp232, Trp365, and the O6 oxygen of terminal Gal of lactose. Although glycerol is a small molecule compared with NeuAc, NeuAc was expected to bind to $\Delta 16pspST6$, including to the glycerol-binding area.

Superimposition of the substrates-binding sites of the two enzymes

Very recently, the crystal structure of $\alpha$2,3-sialyltransferase ($\Delta 24PmST1$) complex with CMP and lactose was reported (Ni et al. 2007). As a result of having compared crystal structure of $\Delta 16psp26ST$ with those of $\Delta 24PmST1$, it is clearly demonstrated in this study that $\Delta 16psp26ST$ complex with CMP and lactose takes the closure form that has been reported in $\Delta 24PmST1$. However, as shown in Figure 2D, a smaller conformational difference between $\Delta 16pspST6$ and the $\Delta 24PmST1$ is observed. In the case of $\Delta 16pspST6$, the side chain of Asn174 located in the substrate-binding cavity may form hydrogen bond with lactose through the interaction with the O1 hydroxyl group of glucose residue. However, in the case of $\Delta 24PmST1$, the Asp84 amino acid residue which corresponds to the Asn174 of $\Delta 16pspST6$ in the primary amino acid sequence might not interact with all the hydroxyl group of lactose, but the side chain of Asn85 amino acid residue of $\Delta 24PmST1$ might form hydrogen bond with the lactose through the interaction with the O3 hydroxyl group of Gal.

Proposed catalytic mechanism

In general, the mechanism of inverting glycosyltransferases is thought to be similar to the one of the inverting glycosylhydrolases with the requirement of one acidic amino acid that activated the acceptor hydroxyl group by deprotonation (Lairson and Withers 2004). The reaction catalyzed by $\Delta 16pspST6$ is the transfer of NeuAc from CMP-NeuAc to the O6 oxygen of the terminal Gal of lactose. A reasonable mechanism involves attack by a deprotonated form of the incoming 6-hydroxyl group of the acceptor lactose or oligosaccharide, on the C2 position of the
sialic acid (Figure 3). Formation of a sialic acid α2,6-linkage and subsequent dissociation of the CMP molecule follows. This reaction would require a catalytic base to deprotonate the hydroxyl group at the C6 position of Gal. Atom OE2 of Asp232 is located 2.7 Å from the nucleophilic O6 of the 6-hydroxyl group. This suggests that Asp232 could play a key role as the catalytic base in the transfer reaction. His405 was located near the phosphate oxygen of CMP. His405 may act as a catalytic residue by protonating phosphate oxygen as a catalytic acid or by stabilizing the transition state to interact with sialic acid in this state. Both amino acid residues, Asp232 and His405, of the Δ16pspST6 are conserved as Asp141 and His311 in the Δ24PmST1. The Δ16pspST6 structure described here supports the proposed catalytic mechanism for CMP-NeuAc-dependent sialyltransferases. All structural and kinetic evidence for inverting glycosyltransferases thus far supports a direct displacement mechanism through an oxocarbenium-ion-like transition state with general base assistance. Because the α2,6-sialyltransferase is inverting glycosyltransferase, we can expect that a general base is needed for the catalytic reaction. In the crystal structure of Δ16pspST6, only Asp232 was within hydrogen-binding distance of the acceptor oxygen (position 6 of the galactose residue in the acceptor substrate lactose) and no other atom in the protein could interact with position 6 of the lactose. Furthermore, Asp232Asn mutants show dramatically decreased sialyltransferase activity. From these results, it is expected that Asp232 of Δ16pspST6 might act as a catalytic base. Site-directed mutagenesis experiments were performed to help determine the role of residues Asp232, His405, and Trp365. The results of the mutagenesis study are summarized in Table II. Point mutations in three residues dramatically reduced the enzymatic activity, suggesting that Asp232, His405, and Trp365 are essential for sialyltransferase activity.

Although Δ24PmST1 has sialyltransferase activity with not only α2,6 linkage but also α2,3 linkage (Ni et al. 2007), in the crystal structure of the Δ24PmST1 complex with CMP and lactose, lactose binds as an α2,3 productive complex with Δ24PmST1. The distance between O3 of the Gal residue of lactose and the CMP phosphate oxygen is 4.7 Å. In the crystal structure of inverting glycosyltransferase GlcAT-1 with uridine di-phosphate (UDP) product and the acceptor sugar-binding complex, the corresponding distance between acceptor oxygen and UDP β-phosphate oxygen is 5.1 Å (Pedersen et al. 2000). In the crystal structure of Δ16pspST6, the corresponding distance between the acceptor O6 oxygen Gal residue of lactose and the CMP phosphate oxygen was 4.7 Å. These three distances are similar and reasonable values for glycosyltransferase activity. These results also support the proposed reaction mechanism.

### Proposed determination of α2,6 reaction specificity

As a result of having compared superimposition the lactose-binding areas of the Δ16pspST6 and Δ24PmST1 complexes with CMP and lactose, O3 oxygen of Gal in the Δ24PmST1 complex with CMP and lactose and O6 oxygen of Gal in the Δ16pspST6 complex with CMP and lactose stay in the almost same position (Figure 2D). In the case of Δ24PmST1, lactose locates 90° to the direction that turned mainly on acceptor oxygen atom compared with those of Δ16pspST6.

In the crystal structure of the Δ24PmST1 complex with CMP and lactose, lactose was recognized by only five residues: Trp270, Asp141, His112, Asn85, and Met144. All the residues contacted only the Gal part of lactose. Three of the five residues (Trp270, Asp141, and His112) are conserved in Δ16pspST6 (Trp365, Asp232, and His204 respectively) and structurally are identical positions. Asn85 and Met144 in Δ24PmST1 correspond to His175 and Ala235 in Δ16pspST6, respectively. His175 and Ala235 in Δ16pspST6 did not interact with lactose. The main difference in the Gal-binding site is the presence of His123 in Δ16pspST6 and Asn85 in Δ24PmST1. The His123 in Δ16pspST6 is within hydrogen-bonding distance of O3 and O5 of the Gal part and is expected to determine the orientation of the Gal part of the lactose. The corresponding residue in Δ24PmST1 is Pro34, which cannot form a hydrogen bond.
with the Gal part. Asn85 in Δ24PmST1 is within hydrogen-bonding distance of O6 of Gal and is expected to determine the orientation of the Gal part of lactose. The corresponding residue in Δ16pspST6 is His175, which might not make a hydrogen bond with O6 of the Gal part. Therefore, we expect that His123 in Δ16pspST6 is the structural contributing factor. The α2,6 reaction specificity of Δ16pspST6 is likely to be determined by His123. We propose that the lactose-binding sites of Δ16pspST6 and Δ24PmST1 are similar except that His123 in Δ16pspST6 can determine the orientation of the Gal part of lactose. Without His123, the enzyme can bind lactose in two ways: one in the Δ16pspST6–CMP–lactose complex structure and the other in the Δ24PmST1–CMP–lactose complex structure. This phenomenon can be explained by the fact that Δ16pspST6 produces only an α2,6 linkage product, whereas Δ24PmST1 can produce not only α2,6 but also α2,3 linkage products.

General discussion

Our structural analysis has revealed a basis for understanding NeuAc transfer to the acceptor substrate. The structure of Δ16pspST6 is highly similar to that of Δ24PmST1 in terms of the tertiary fold and CMP binding. Insightfully, the catalytic base Asp232 of Δ16pspST6 can be superimposed upon Asp141 of the Δ24PmST1 structure. These data suggest that the reaction mechanism and the catalytic site structure may also be applicable to other sialyltransferase families. In the CAZY database, Δ24PmST1 is classified into GT80 family and Δ16pspST6 may also be classified into GT80 family according to its amino acid sequence. On the other hand, Cst-I and Cst-II are classified into GT80 family. Therefore, it is expected that the reaction mechanism of these enzymes is almost same, but the amino acid residues which may act as general base residues which may act as general base are thought to be different between GT24 and GT80 family.

There are no overall sequence similarities between Δ16pspST6 and sialyltransferases in mammals. Mammalian sialyltransferases have several conserved sequences, named sialyl motifs L, S, and VS (Wen et al. 1992; Sasaki 1996). Sialyl motif VS has His and Glu in its sequence. In the case of human α2,3-sialyltransferase, it has been demonstrated that Glu321Gln mutants show decreased sialyltransferase activity (Jeanneau et al. 2004). So, the conserved Glu and His residues (VS motif) in mammalian sialyltransferases, Glu must be replaced by Asp in Δ16pspST6, may play the almost same catalytic roles as Asp232 and His405 in Δ16pspST6.

In conclusion, we have presented the results from a study of the crystal structure of the ternary complex of Δ16pspST6/CMP/lactose and a mutational analysis. A comparison of the structure of Δ16pspST6 with the previously published structure of Δ24PmST1 had identified several amino acid residues that may act to direct the substrate specificity of sialyltransferases. Our results have not enabled us determine the precise molecular details of the substrate specificity differences between the sialyltransferases, but they have provided valuable structural information toward a comprehensive understanding of these enzymes.

Material and methods

Cloning, expression, purification, and crystallization of recombinant α2,6-STase

The cloning, expression, purification, and crystallization of the putative mature-form recombinant of α2,6-sialyltransferase produced by Photobacterium sp. JT-ISH-224, Δ16psp26ST, have previously been reported (Okino et al. 2007). In brief, crystallization of the enzyme was carried out as follows. Single crystals were grown by the hanging-drop vapor diffusion method at 20°C. The protein solution was composed of 5 mg/mL Δ16psp26ST in 50 mM Tris–HCl (pH 7.5) containing 10 mM CMP and 10 mM lactose. The well solution contained 200 mM lithium sulfate and 30% (w/v) polyethylene glycol 4000 in 0.1 M Tris–HCl (pH 8.5) buffer. The drops consisted of 1 µL of protein solution and 1 µL of well solution (Okino et al. 2007).

Sialyltransferase assay

The 30 µL reaction mixture contained of enzyme solution, 120 mM lactose, 2.3 mM CMP-NeuAc (Nakarai, Kyoto, Japan), 4620 Bq CMP-[4,5,6,7,8,9-14C]-NeuAc (Amershams Biosciences, Uppsala, Sweden), 20 mM bis-Tris buffer (pH 6.0), 0.5 M NaCl and 0.03% Triton X-100. The reaction was carried out at 30°C in duplicate. After reaction, the reaction mixture was diluted with 5 mM potassium phosphate buffer (pH 6.8) to 2 mL, and applied to a column of AG1-X2 resin (0.5 × 2 cm, Bio-Rad Laboratories, Hercules, CA). The eluate (2 mL) was collected and added with the scintillation cocktail. The mixture was measured directly into a liquid scintillation counter (model TR 1900, Packard). One unit (U) of sialyltransferase activity was defined as the amount of enzyme that transferred 1 µmol of NeuAc per min to lactose at pH 5.0 and 30°C.

X-ray data collection, structure solution, and refinement

Before data collection, crystals were transferred stepwise into a cryoprotectant solution containing 10% glycerol under crystallization conditions (100 mM Tris–HCl (pH 8.5), 50 mM Tris–HCl (pH 7.5), 200 mM lithium sulfate, 30% (w/v) polyethylene glycol 4000, 10 mM CMP and 10 mM lactose) and then flash-frozen at 100 K using a cryo-system (Rigaku, Tokyo, Japan). X-ray diffraction data were collected using a Jupiter 210 (Rigaku/MSC Corporation) and the synchrotron radiation (1.000 Å wavelength) at the beamline BL38B1 of Spring-8 (Hyogo, Japan). The data were processed using HKL2000 (Otwinowski 1993) and the data statistics are summarized in Table III. The crystal of Δ16psp26ST in a complex with CMP and lactose (α2,6-STase/CMP, lactose) diffracted up to 2.5 Å, and belonged to the primitive hexagonal space group P3121 with unit cell dimensions of a = b = 90.29 Å and c = 204.33 Å. The crystal structure of Δ16psp26ST/CMP, lactose was determined by molecular replacement using Δ24PmST1 (Ni et al. 2007) as a search model with the program Molrep (Vagin and Teplyakov 1997). Structure refinement was done with the program Refmac (Murshudov et al. 1997) with diﬀraction data from 21.4 to 2.5 Å. After the first refinement step, the atomic model of the protein was rebuilt with the program Coot (Emsley and Cowtan 2004). Iterative cycles of refinement and manual rebuilding in Coot were carried out until the R_pwr factor is 19.2% and the R_free factor is 24.3%. Stereochemical checks were carried out

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with PROCHECK (Vaguine et al. 1999). The atomic coordinates of Δ16psp26ST have been deposited in the Protein Data Bank under accession code 2Z4T.

Construction, expression, and purification of truncated sialyltransferase (Δ109pspST6)

The PCR reaction mixture of 50 μL consisted of 500 ng of the template DNA fragment N1C0/pTrc99A, encoding Δ16psp26ST, 50 pmol of each primer (N3Bsp: 5′-AAGTACATGACGTCATGGGCTCCATTGTAA-3′ and C0BamHi: 5′-TTTTTTGGATCCCTAGACTGCACTGGTACGTTAACATGAA-3′), 4 μL of 2.5 mM each dNTP, 2.5 units of PyroBest DNA polymerase (0.5 μL) and 5 μL of 10 × PyroBest buffer II (Takara Biochemicals, Shiga, Japan), in accordance with the manufacturer’s instructions. The reaction was hot-started at 96°C for 3 min, incubated at 96°C for 1 min, 55°C for 1 min, and 72°C for 2 min for five cycles, and then further incubated at 72°C for 6 min in a Program Temp Control System (ASTEK, Fukuoka, Japan). The PCR product was cloned into a pCR4TOPO vector (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. The pCR4TOPO vector containing the PCR products was introduced into E. coli TB1.
as templates. The third PCR products were inserted into the pCR4-TOPO (Invitrogen) vector and sequenced via cycle sequencing using an ABI 3100-Avant DNA analyzer (Applied Biosystems, Foster City, CA) in accordance with the manufacturer’s instructions. These DNA fragments were cloned between the NcoI and BamHI sites of the pTRe99A vector. Expression of mutant proteins was carried out by the methods described in the above section.

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**Conflict of interest statement**

None declared.

**Abbreviations**

CAZy, carbohydrate-active enzymes; CMP, cytidine monophosphate; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GT-B, glycosyltransferase-B; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NeuAc, N-acetylneuraminic acid; PCR, polymerase chain reaction; STase, sialyltransferase; UDP, uridine di-phosphate.

**References**


