Unique gangliosides synthesized in vitro by sialyltransferases from marine bacteria and their characterization: ganglioside synthesis by bacterial sialyltransferases

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Abstract On the basis of the results outlined in our previous report, bacterial sialyltransferases (ST) from marine sources were further characterized using glycosphingolipids (GSL), especially ganglio-series GSLs, based on the enzymatic characteristics and kinetic parameters obtained by Line weaver-Burk plots. Among them, GA1 and GA2 were found to be good substrates for these unique STs. Thus, new gangliosides synthesized by α2-3 and α2-6STs were structurally characterized by several analytical procedures. The ganglioside generated by the catalytic activity of α2-3ST was identified as GM1b. On the other hand, when enzyme reactions by α2-6STs were performed using substrates GA2 and GA1, very unique gangliosides were generated. The structures were identified as NeuAcα2-6GalNAcβ1-4Galβ1-4Glcβ-Cer and NeuAcα2-6Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-Cer, respectively. The synthesized ganglioside NeuAcα2-6 GalNAcβ1-4Galβ1-4Glcβ-Cer showed binding activity to the influenza A virus {A/Panama/2007/99 (H3N2)} at a similar level to purified sialyl(α2-3)paragloboside (S2-3PG) and sialyl(α2-6)paragloboside (S2-6PG) from mammalian sources. The evidence suggests that these STs have unique features, including substrate specificities restricted not only to lacto-series but also to ganglio-series GSLs, as well as catalytic potentials for ganglioside synthesis. This evidence demonstrates that effective in vitro ganglioside synthesis could be a valuable tool for selectively synthesizing sialic acid (Sia) modifications, thereby preparing large-scale gangliosides and permitting the exploration of unknown functions.— Kamimiya, H., Y. Suzuki, T. Kasama, H. Kajiwara, T. Yamamoto, T. Mine, S. Watarai, K. Ogura, K. Nakamura, J. Tsuge, and Y. Kushi. **Unique gangliosides synthesized in vitro by sialyltransferases from marine bacteria and their characterization: ganglioside synthesis by bacterial sialyltransferases. *J. Lipid Res.* 2013.** 54: **571–580.**

Supplementary key words ceramides • influenza A virus • glycolipids •mass spectrometry • membranes

Glycosphingolipids (GSL) are amphipathic molecules consisting of a hydrophilic sugar chain and a hydrophobic ceramide moiety. They are usually located in the outer leaflet of the cell membrane, in which they are anchored by the ceramide portion. The carbohydrate chains are directed toward the cell exterior, have enormous structural diversity, and constitute part of the glycocalyx network on

Abbreviations: Cer, ceramide; CHAPS, 3-[(3-cholamidopropyl) dimethylammonium]-1-propanesulfonic acid; CMP-NeuAc, cytidine-monophospho-5′-*N*-acetylneuraminic acid; GSL, glycosphingolipid; LOS, lipooligosaccharide; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PMAA, partially permethylated alditol acetate; PG, nLc_4Cer (paragloboside), neolactotetraosylceramide; PVDF, polyvinylidene difluoride; S2-3PG, sialyl(α2-3)paragloboside; S2-6PG, sialyl(α2-6)paragloboside; SI-MS, secondary ion mass spectrometry; Sia, sialic acid; ST, sialyltransferase; TCID50, 50% of tissue-culture infectious dose.

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the cell surface (1). They have been implicated in a variety of phenomena, including cell-cell recognition, neurite outgrowth, synaptogenesis, transmembrane signaling, cellular growth and differentiation, and oncogenic transformation (2–5). Gangliosides are GSLs containing one or more sialic acid (Sia) residues and play important roles in a variety of biochemical phenomena, such as cell-cell recognition and viral infection (6). Gangliosides are synthesized by sialyltransferases (ST) that transfer Sia from cytidinemonophospho 5′-*N*-acetylneuraminic acid (CMP-NeuAc) to the nonreducing terminal of glycoconjugates (7, 8).

During the past decade, considerable evidence has been accumulated showing altered GSLs and ganglioside content and profile in tumor tissues and transformed cultured cells. These changes are frequently accompanied by alterations in glycosyltransferase activities, which are involved in the synthesis of these tumor-specific antigens (6–8). Many reports have described the detection of glycoconjugates on cell surfaces and in tissues using a very small amount of sample. Detailed structural characterization of GSLs is a prerequisite for understanding the relationship between their chemical structures and functions in cellular activities (9–11).

Recently, several groups have discovered and characterized the features of STs from *Campylobacter jejuni* and *Neisseria meningitidis*, which are gram-negative bacteria (12), and using the potential ST activities, in vitro ganglioside syntheses have been achieved by practical scale preparations (13–15). In a previous report, we showed the in vitro unique ganglioside synthesis by recombinant STs from marine bacterial sources; moreover, we showed that the synthesized gangliosides showed binding activity to the influenza A virus [A/Panama/2007/99 (H3N2)] at a similar level to purified sialyl(α2-3)paragloboside (S2-3PG) and sialyl(α2-6)paragloboside (S2-6PG) from mammalian sources. Thus, STs from marine bacteria have been found to catalyze efficiently the in vitro sialylation of GSLs as well as oligosaccharide, especially lacto-series ganglioside synthesis (16). These simple and less time-consuming ganglioside preparations on a large scale may have strong potential for preparing natural ligands, such as gangliosides, and for promoting a better understanding of the biological roles of GSLs. In this study, we applied these ST enzymes for unique ganglioside synthesis, especially ganglio-series ganglioside preparations, and characterized the obtained structures.

MATERIALS AND METHODS

Materials

Ganglio-series gangliosides (GM2, GM1a, GD1a, and GD1b) were purified from bovine brains as described previously (17). GA1 was purified from GM1a by acetic acid treatment (18) and GA2 was purified from Tay-Sachs brain, which was kindly supplied by Dr. M. Owada (Nihon University School of Medicine). PG, sialyl(α2-3)paragloboside (S2-3PG) and sialyl(α2-6)paragloboside were prepared from human erythrocytes and human meconium.

Anti-GM1b monoclonal antibody (mAb) GG51 (IgG) was kindly provided by Kotani et al. (19). STs isolated from marine bacteria, including #1 (ish-224, 05JTC1), #2 (ish-467, 05JTD2), and #3 (faj-16, 05JTE1), which form α2-3 Sia linkages named α2-3STs, as well as #4 (ISH-224, N1C0), #5 (pda-rec, 05JTB2), and #6 (pda-0160, 05JTA2), which form α2-6 Sia linkages named α2-6STs, were obtained from the Glycotechnology Business Unit, Plant Innovation Center, Japan Tobacco Inc., and characterized (20, 21). These are all recombinant enzymes and detailed substrate specificities were already confirmed using pyridylamidated saccharide derivatives (21, 22). Nonionic detergents, Triton X-100, CHAPS, Brij, deoxycholate, octylglycoside, and Triton CF-54 were used in the ST assay. All detergents except for deoxycholate are the products of Sigma Ltd. (St. Louis, MO).

ST assay using GSLs as acceptor substrates

Assays for the enzyme activity were performed as follows: 3.33 mM CMP-NeuAc, 0.3 mM acceptor substrate (GSLs), 0.3% (w/v) Triton X-100, 10 mM $MnCl_2$, 333 mM sodium cacodyrate (pH 6.5), and 500 mM NaCl were used for a standard method. Each ST was added as 2 μl of enzyme solution (0.1 U) to the incubation mixture (16). One unit (U) was defined as the amount of enzyme that converted 1 μmol NeuAc per minute to lactose, as described previously (22). GSLs were dissolved in chloroform/methanol (2:1 by vol), dried under nitrogen stream, and then suspended in the rest of the reagents used for the assay described above. An aliquot of the total volume (30 μl) was incubated at 37°C for the required incubation times. Alternative ST assays were also performed by changing temperatures and pHs to investigate the optimal conditions for these STs. The reaction was terminated by adding water (0.5 ml) and application of a Sep-pak C18 cartridge (Waters, Milford, MA), which was equilibrated with methanol and distilled water. After washing the column with water to remove the nonreacted CMP-NeuAc and the salts, reaction mixtures with 2 ml of distilled water, 1 ml of methanol, and 1 ml of chloroform/methanol (2:1 by vol) were added. The lipid fractions eluted with the chloroform/methanol mixture were evaporated, and an aliquot was used for high-performance thin-layer chromatography (HPTLC) and TLC/immunostaining (16). Ganglioside production on TLC was calculated quantitatively using NIH Image J software (23).

Large scale ganglioside preparations

For a large-scale synthesis, the volume of the incubation mixtures was scaled up to 500 μl. Six to ten microliter enzyme solution (0.3 to 0.6 U) were added to the ST assay solution, and incubated under the appropriate conditions. After preparations, reverse phase chromatography by a Sep-pak C18 cartridge and ion exchange chromatography by DEAE-Sephadex A-25 (acetate form) were done and confirmed to be a pure form on TLC. Synthesized gangliosides were used for the influenza virus binding assay after GC/MS and NMR analyses.

Kinetics parameters analyses

Enzyme characteristics and kinetics analyses were done as previously described (16). The apparent kinetics of ST interactions with the acceptor substrates was determined using a saturating CMP-NeuAc. Data points represented the average of at least duplicate values.

HPTLC and TLC/immunostaining

HPTLC and TLC/immunostaining of GSLs were as previously described with slight modifications (17). A HPTLC plate (silica gel 60 HPTLC, Merck, Darmstadt, Germany) was used with the following solvent systems: (I) chloroform/methanol/water (60:35:8 by vol) and (II) chloroform/methanol/water (55:45:10 by vol) containing 0.02% $CaCl_2$. GSLs on TLC plates were visualized

with orcinol-H_2SO_4 (24) or resorcinol-HCl (25). The developed and dried TLC plates were soaked for 1 min in a 0.02% solution of polyisobutylmethacrylate (Tokyo Kasei Kogyo, Japan) dissolved in hexane and allowed to air-dry, and then blocked by incubation in 1% BSA/phosphate-buffered saline (PBS) at 37°C for 30 min. They were then rinsed five times with PBS/0.05% Tween 20 washing buffer (Wako Pure Chemical Industries Ltd.) and incubated with mAbs at a dilution of 100 mg/ml with PBS at 4°C overnight. After washing, the plates were reincubated with horseradish peroxidase-conjugated goat anti-mouse IgM antiserum (Cappel Laboratories, West Chester, PA) at 37°C for 2 h. As a final step, they were visualized with peroxidase substrate solution (immunostain kit, Konica, Tokyo).

Mass spectrometry analysis of gangliosides synthesized by STs

Sialylated products were analyzed by in situ polyvinylidene difluoride (PVDF) membrane (ATTO, Tokyo, Japan) transfer followed by secondary ion mass spectrometry (SI-MS), as described previously (26, 27). GSLs developed by TLC were transferred to a PVDF membrane by TLC blotting, after which GSL bands on the membrane were developed with primurin spray (28), excised, and placed on a mass spectrometer probe tip, with a few microliters of triethanolamine added as the matrix.

Permethylation analysis and neuraminidase treatment

After separating the products from the reaction mixtures containing nonreacted substrates with a small DEAE-Sephadex (acetate form) column (4 cm × 10 mm), the synthesized gangliosides were confirmed by permethylation analysis. Partially methylated alditol acetates of the synthesized gangliosides were prepared; detailed conditions of GC/MS analysis were as previously described (29–32). Neuraminidase digestion of the newly synthesized gangliosides was performed in acetate buffer (50 mM, pH5.5) containing neuraminidase of *Arthrobacter ureafaciens* (Nacalai Tesque, Kyoto, Japan) at 37°C for 3 h. The reaction mixture was then extracted with 2 ml of chloroform/methanol (2:1 by vol). The extracts also underwent permethylation analysis after confirming the digestion of synthesized ganglioside by TLC.

Binding of influenza A virus to gangliosides

Madin-Darby canine kidney cells (MDCK) were cultured in Dulbecco's Modified Eagle's medium containing 10% fetal calf serum. Influenza virus A/Panama/2007/99 (H3N2) was used for this assay. The titer of infectious virus was determined by limiting dilution in cultures of MDCK cells and is expressed as 50% of the tissue-culture infectious dose (TCID50) (33). Five hundred microliters of 1,000 TCID50 virus was mixed 1:1 (v/v) with ganglioside-containing liposome suspensions and incubated at 37°C for 1 h. The liposomes were adjusted to the concentration of the standard and synthesized gangliosides (0, 50, 100, 150, and 250 μg) (34). Virus-induced cytopathic effects were then monitored by light microscopy. The virus titer was obtained as the reciprocal of the highest dilution giving TCID50.

RESULTS

Effects of various detergents for ST assay

We successfully used the detergent (Triton X-100) for ST assay in a previous study (16). To provide much higher yields of ganglioside products in the assay, we investigated the optimal conditions using different detergents, such as nonionic detergents Brij, deoxycholate, octylglycoside, and Triton CF-54. Among them, octylglycoside showed the best and most increased percentage of ganglioside synthesis, which was 176% compared with using Triton X-100. However, there were unknown spots on the TLC plate and the spots comigrated with the ganglioside products, which disturbed the precise quantitation of the products. As it was impossible to remove the spots even after use of a C18 reversed-cartridge column, Triton X-100 was used in experiments, and increased yields of about 250% of new ganglioside syntheses by octylglucoside and deoxycholate were obtained (data not shown here).

Sialylation of GA1 and GA2 by marine STs

The results of ganglioside synthesis using GA1 and GA2 as substrates by using STs from marine bacteria are shown in **Fig. 1**. STs showed affinity to substrates, although there were some quantitative differences between STs. All bands appeared newly on TLC plates after ST enzyme reactions occurred with resorcinol-HCl reagent (data not shown). All gangliosides synthesized by α2-3STs migrated to below the position of standard GM1a (Fig. 1A, lanes 3–5), and gangliosides synthesized by α2-6STs migrated even further, that is, below the position of gangliosides synthesized by α2-3STs (Fig. 1A, lanes 6–8). In the case of substrate GA2, the ganglioside synthesized by α2-6STs migrated further below the RF value of GM2 ganglioside on TLC (Fig. 1B, lanes 6–8). In contrast, no gangliosides were synthesized by α2-3STs even after longer incubation periods or excess use of α2-3STs compared with the normal assay conditions described in the text (Fig. 1B, lanes 3–5). No other bands except the predicted gangliosides and unreacted substrates were detected in any lane. After neuraminidase treatment of the synthesized gangliosides, all Rf values were shifted back to the original position of each substrate

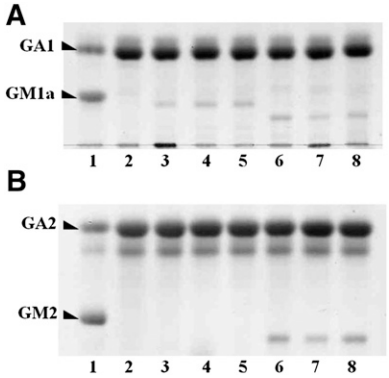

Fig. 1. Sialylation of GA1 and GA2 by marine STs. After incubation at 30°C for 3 h, lipid fractions were obtained from enzyme assay mixtures (30 μl) as described in the text. One fifth of the lipid fractions was applied to TLC, developed with solvent system I, and visualized using orcinol/H_2SO_4 reagent (25). GA1 (A) and GA2 (B) were used as substrate GSLs. (A, B) Lane 1 is the standard GSLs: GA1 and GM1a from the top (A) and GA2 and GM2 from the top (B). In lanes 3–5, #1, #2, and #3α2-3ST were used, respectively. In lanes 6–8, #4, #5, and #6α2-6ST were used. Lane 2 represents TLC without each STs. STs were added in the assay mixtures (2 μl of 0.1 U ST).

(GA1 and GA2) on TLC, confirming the presence of Sia residues in the new synthesized gangliosides (data not shown).

Enzyme characteristics and kinetic analyses

No significant differences were observed between #1–#3STs and #4–#6STs on the enzymatic characteristics. Therefore, #3 as α2-3ST and #4 as α2-6ST were chosen and used in the following study. The reaction products synthesized by both STs were proportional to the incubation time up to 30 min and reached a plateau (**Fig. 2**). On the other hand, at 30°C, all STs showed higher enzyme activities than at 37°C (1.2- to 1.5-fold increase) except for #4ST with GA1 acceptor (**Table 1**). To further investigate the mechanism of these STs, Line weaver-Burk plots were generated and individual K_m and V_{max} values were obtained (Table 1). #3ST, which catalyzed the formation of α2-3linked Sia, showed a high K_m value to GA1 acceptor substrate, whereas the K_m value of #4ST, which catalyzed the formation of α2-6linked Sia, was 0.381 mM. Similarly, #4ST to GA2 substrate exhibited almost the same value (0.396 mM) to GA2 as the case of GA1 and the lowest V_{max}/K_m value among them. These enzyme kinetic parameters were comparable to results in our previous study. To clarify whether GA1 and GA2 are equivalently used as substrates to the lacto/neolacto-series GSLs, enzyme reactions under the same pH, incubation time, and temperatures were performed. The syntheses of all ganglioside products were almost proportional to the added STs (0.01 to 0.4U) in the assay, as shown in Fig. 2C. In the case of nLc_4Cer as substrate, both #3 and #4STs synthesized about two times more sialyl(α2-3)paragloboside (S2-3PG) and sialyl(α2-6)paragloboside products than did GA1 and GA2 as substrates (Fig. 2B, C).

Structural elucidation by SI-MS spectrometry after TLC blotting

Fig. 1 shows that #3ST catalyzed the formation of α2-3linked Sia, while #4ST catalyzed the formation of α2-6linked Sia using substrates. Typical gangliosides synthesized by #3 and #4STs were temporally designated as S2-3GA1, S2-6GA1, and S2-6GA2, respectively, and were structurally elucidated in more detail. First, gangliosides S2-3GA1 and S2-6GA1 were analyzed by in situ PVDF membrane transfer, followed by SI-MS; the results are shown in **Fig. 3**. In the negative ion SI-MS spectra of gangliosides S2-3GA1 and S2-6GA1, the major pseudo-molecular ions $[M\text{-}H]^-$ were detected at *m/z* 1545.7 and 1573.7, which were consistent with values calculated for the proposed structures with C18:0-C20 sphingenine and C18:0-C18 sphingenine in both spectra (Fig. 3B, C). This finding indicates the presence of sugars with the same composition in S2-3GA1 and S2-6GA1. Furthermore, the ions responsible for successive elimination of sugar moieties with ceramide portions were detected at *m/z* 1253.8 and 1281.7 for $[M\text{-}H\text{-}NeuAc]^-$, at *m/z* 1091.8 and 1119.6 for $[M\text{-}H\text{-}NeuAc\text{-}Gal]^-$, and at *m/z* 888.5 and 916.4 for $[M\text{-}H\text{-}NeuAc\text{-}Gal\text{-}GalNAc]^-$, as well as for pseudo-molecular ions.

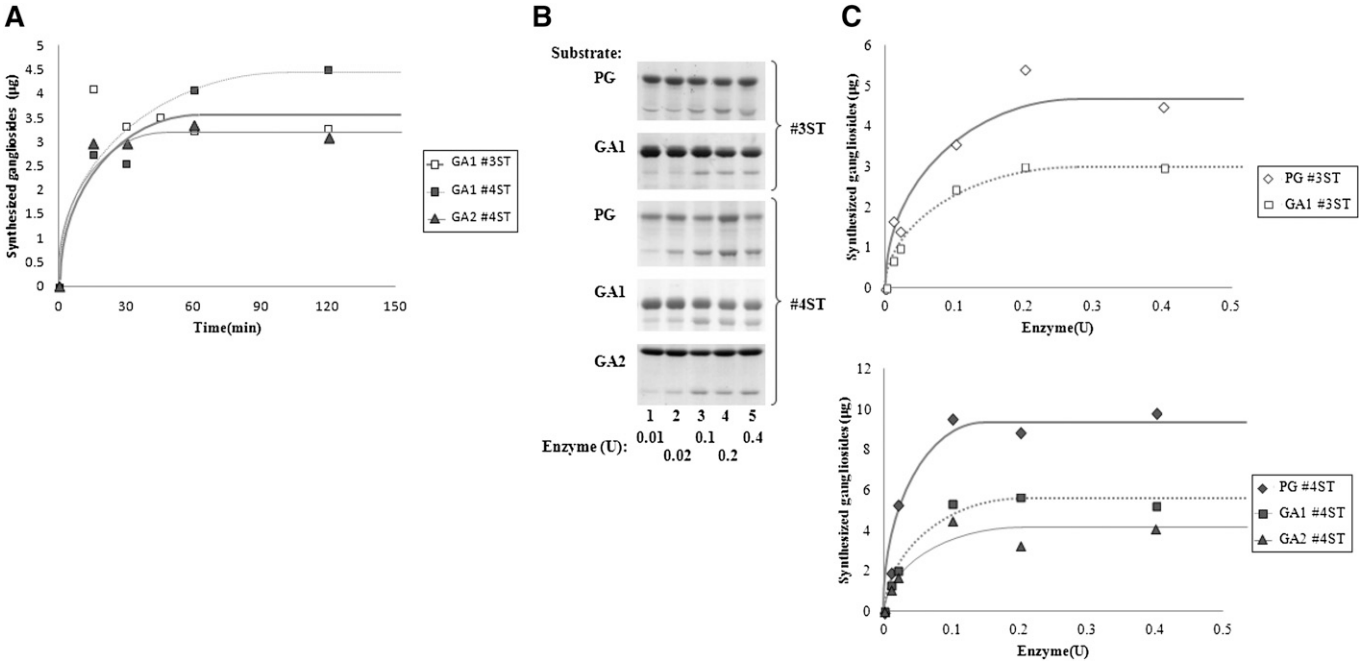

Fig. 2. Incubation time course profiles. After enzyme reactions using the indicated incubation times, lipid fractions purified by Sep-Pak C18 cartridge were chromatographed on TLC. The synthesized gangliosides were quantitated using NIH image software (23) and calibrated with plots of standard gangliosides. The incubation times used for enzyme reactions are shown in the horizontal plot, and the products of synthesized gangliosides in the reaction are shown in the vertical plot. (A) Profiles of gangliosides by #3ST (S2-3GA1) (open squares), #4ST (S2-6GA1) (filled squares), and #4ST (S2-6GA2) (filled triangles). (B) Comparative TLC profiles of the synthesized ganglioside by #3ST and #4ST using neolacto- and ganglio-series substrates at 30°C. (C) Results of quantitative analyses of individual synthesized gangliosides using the different units of ST (top, #3ST; bottom, #4ST; open/filled diamonds, nLc_4Cer; open/filled squares, GA1; filled triangles, GA2). Synthesized gangliosides and the units of ST used are shown in the horizontal and the vertical plots, respectively.

TABLE 1. Kinetics parameters of STs

Enzyme	Substrate	K_m (mM)	V_{max}	V_{max}/K_m	Percentage of Synthesized Gangliosides (%) 30°C	37°C
#3	GA1	1.048	3.499	3.3387	155.09	100
#4	GA1	0.381	0.789	2.0709	99.44	100
#4	GA2	0.396	0.508	1.2828	120.42	100

On the other hand, in the negative SI-MS spectrum of ganglioside S2-6GA2, the major pseudo-molecular ions $[M-H]^-$ were detected at *m/z* 1385.5, 1413.7, and 1467.8. In contrast, the spectra of substrate gave pseudo-molecular ions $[M-H]^-$ at *m/z* 1065.6 and 1093.5 for GA2 (**Fig. 4A**). From the differences in mass units between the two pseudo-molecular ions, the attached sugar residue at the nonreducing terminal of the synthesized gangliosides corresponded to the mass units of NeuAc, that is, 292 (Figs. 3 and 4). This result confirmed that NeuAc from CMP-NeuAc was transferred to the substrates GA1 and GA2 via ST activity.

Reactivity of newly synthesized gangliosides to mAbs specific for GM1b

TLC/immunostaining was carried out using mAbs (**Fig. 5**). Fig. 5B shows the results obtained with antibody GG51, which is directed against GM1b carbohydrate chains. Bands of newly synthesized gangliosides showed a positive reaction with mAb GG51 (Fig. 5, lane 2). These observations clearly indicate that the ganglioside S2-3GA1 synthesized by #3ST has the NeuAcα2-3Gal linkage within the molecule. However, this mAb did not react with S2-6GA1 as reported previously (19).

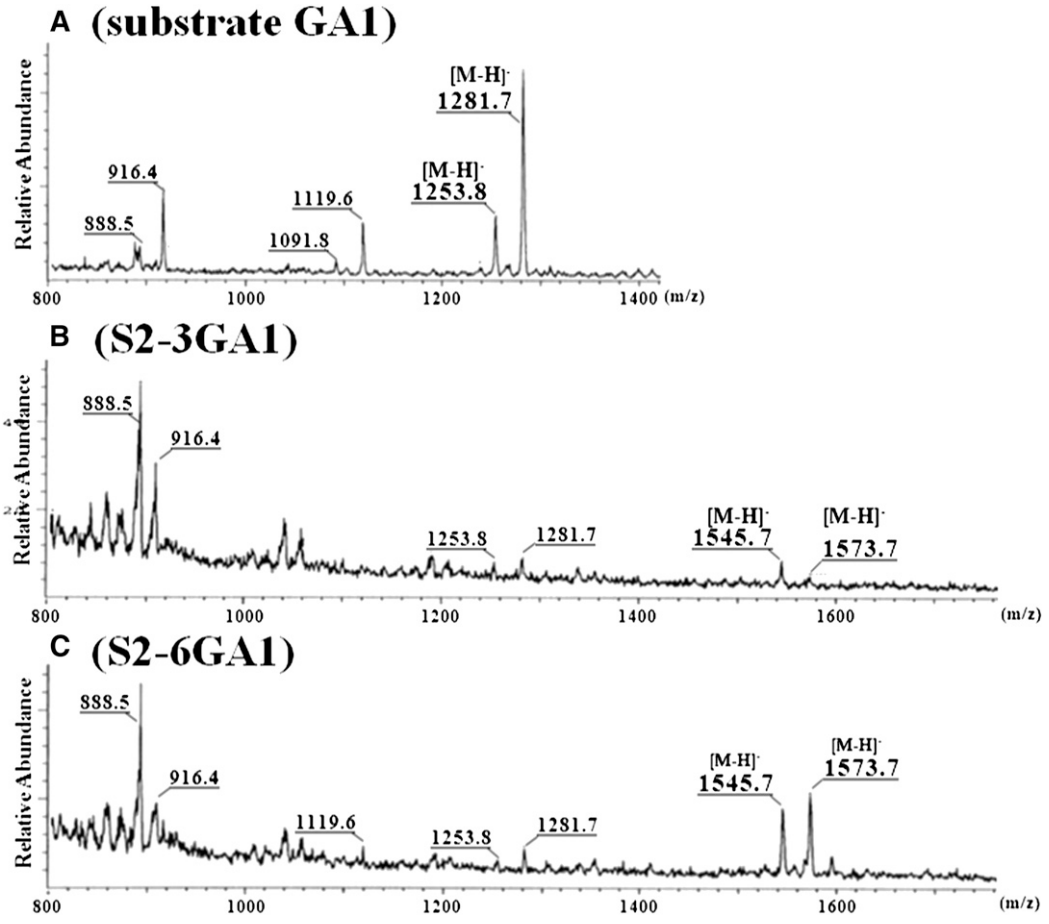

Fig. 3. Mass spectrometric analyses by in situ PVDF membrane transfer of gangliosides synthesized by STs. Enzyme reactions were performed as described in the text with #3 and #4STs. After TLC, PVDF membranes were blotted and GSLs were transferred to the PVDF membrane as described by Taki et al. (26, 27). The blotted PVDF membranes were developed with primurin spray (28) and then analyzed by SI-MS after cutting out the spots representing newly synthesized gangliosides and remaining unreacted substrate. (A) Substrate GA1. (B) Ganglioside synthesized by #3ST (S2-3GA1). (C) Ganglioside synthesized by #4ST (S2-6GA1).

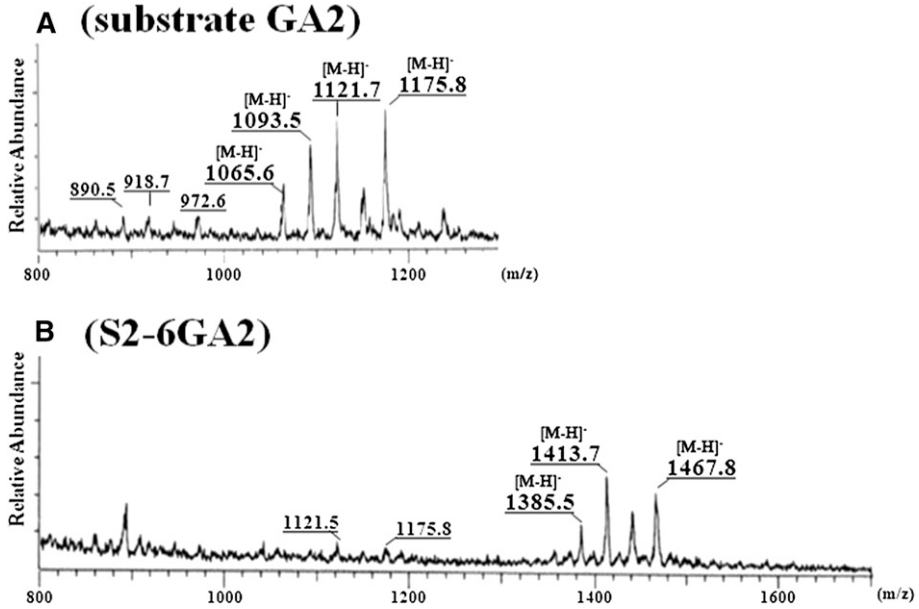

Fig. 4. Mass spectrometric analyses by in situ PVDF membrane transfer of gangliosides synthesized by STs. Enzyme reactions and the mass spectrum analysis were performed as described in Fig. 1. (A) Substrate GA2. (B) Ganglioside synthesized by #4ST (S2-6GA2).

Permethylation analysis of the synthesized gangliosides

Methylation study of the synthesized gangliosides, S2-3GA1, S2-6GA1, and S2-6GA2, was carried out by GC/MS; the result for S2-6GA2 is shown in **Fig. 6**. The individual ion chromatograms of partially permethylated alditol acetates (PMAA) in substrates GA2, GM2, and synthesized S2-6GA2 (Fig. 6A–C) and the mass spectrum of a new peak that appeared for the synthesized ganglioside are shown (Fig. 6D). Substrate GA2 was demonstrated to contain 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol (-4Glc1-) (12.1 min), 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalacitol (-4Gal1-) (12.3 min), and 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-*N*-methylacetoamidogalactitol (terminal GalNAc) (16.7 min) at an approximately equimolar ratio (Fig. 6A). In contrast, GM2 ganglioside was demonstrated to contain 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylglucitol (-4Glc1-) (12.1 min), 2,6-tri-*O*-methyl-1,3,4,5-tri-*O*-acetylgalactitol (-3.4Gal1-) (13.4 min), and 3,4,6-tri-*O*-methyl-1,5-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetoamidogalactitol (terminal GalNAc) (16.7 min) (Fig. 6B). However, on the permethylation analysis of the S2-6GA2 synthesized by #4α2-6ST, the peaks of GC/MS yielded 2,6-tri-*O*-methyl-1,3,4,5-tri-*O*-acetylglucitol (-4Glc1-) (12.1 min), 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol (-4Gal1-) (12.3 min), and a new peak detected at 19.2 min corresponding to 3,4-di-*O*-methyl-1,5,6-tri-*O*-acetyl-2-deoxy-2-*N*-methylacetoamidogalactitol (-6(4)GalNAc1-) (Fig. 6C). In the mass spectrum, a parent ion was observed at *m/z* 392 and fragment ions were present in PMAAs for the parent ion minus HOMe and HOAc (*m/z* 326 and 360) (data not shown here). Furthermore, classical fragment ions assigned in the formulas were also found at *m/z* 116, 158, and 233 (Fig. 6D). The synthesized Sia was determined to be linked at the C-6 position of the terminal N-acetylgalactosamine residue of the substrate GA2 on the basis of the well-characterized substrate specificities of these STs. Further permethylation analysis of the desialylated ganglioside obtained identical peaks to the substrate GA2. On the permethylation analysis of the synthesized gangliosides S2-3GA1 and S2-6GA1, the characteristic peak due to the terminal galactose that appeared in substrate GA1 methylation analysis disappeared, and then new peaks were observed in GC/MS. Those ions corresponded to 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol (-3Gal1-) and 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetylgalactitol (-6Gal1-), showing that Sia was linked at the C-3 or C-6 position of the terminal galactose in the molecule (data not shown).

All the results above indicate that, for synthesized ganglioside structures, S2-3GA1 generated by #3ST was identified as GM1b, whereas the structures of S2-6GA1 and S2-6GA2 generated by #4ST were NeuAcα2-6Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-Cer and NeuAcα2-6GalNAcβ1-4Galβ1-4Glcβ-Cer, respectively. Similarly, identical results

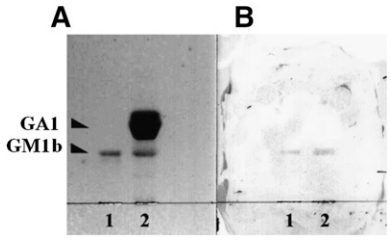

Fig. 5. TLC/immunostaining. Enzyme reactions were performed as described in the text. After TLC development, TLC/immunostaining was performed as previously described with the anti-GM1b mAb (19). Other conditions were identical to those described in Fig. 1. (A) TLC was visualized by orcinol/H_2SO_4. (B) Same TLC procedure used in (A) was performed, followed by TLC/immunostaining. (A, B) In lane 1, standard GM1b was used, and in lane 2, lipid fraction after enzyme reaction was applied. #3α2-3STs were used as STs.

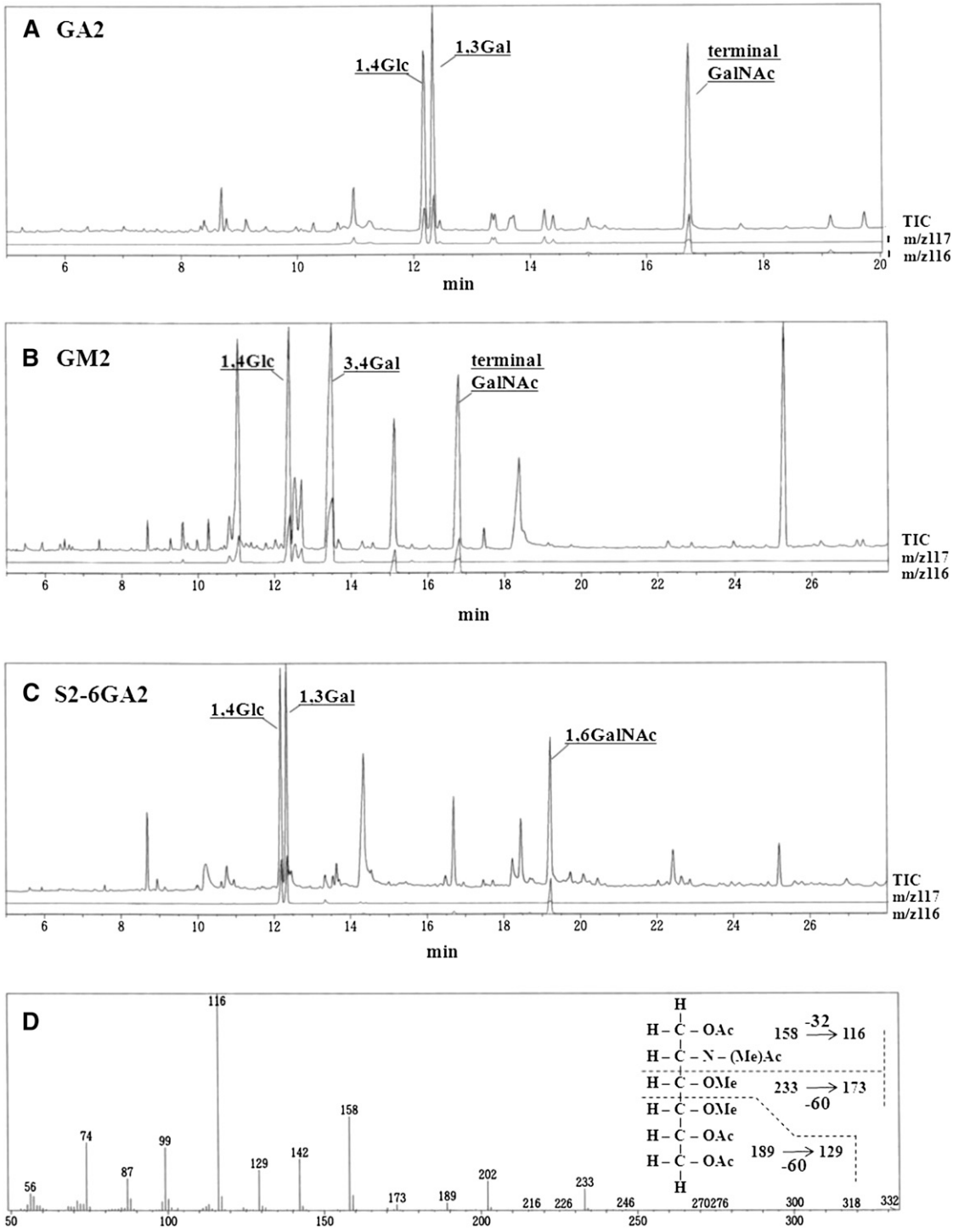

Fig. 6. Permethylation analysis of the synthesized gangliosides. Partially methylated alditol acetate derivatives were prepared from GA2, GM2, and synthesized gangliosides as described previously (29–33). Prepared carbohydrate derivatives were analyzed by GC/MS using the conditions described in the text. Chromatographic profiles of GC/MS and the characteristic mass spectrum of synthesized gangliosides are shown. (A) Substrate GA2. (B) GM2. (C) Ganglioside synthesized by #4α2-6ST (S2-6GA2). (D) Mass spectrum of the peak at 19.2 min in the total chromatographic profile in (C).

were obtained from methylation analysis of the synthesized gangliosides from #1 and #2STs and from #5 and #6STs. Taking these results together, the ganglioside structures generated by #1, #2, and #3STs were the same, and the ganglioside structures generated by #4, #5, and #6STs were the same.

Binding of influenza A virus to gangliosides

To confirm the biological activities of the in vitro synthesized gangliosides, binding to the influenza A virus (H3N2) was compared between synthesized and purified gangliosides from natural sources using liposome methods (**Fig. 7**). The TCID50 value was decreased by standard

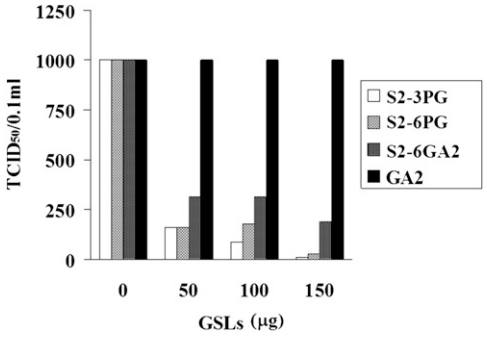

Fig. 7. Binding activity of in vitro synthesized gangliosides to influenza A virus. One hundred microliters of 1,000 TCID50 of the virus [A/Panama/2007/99 (H3N2)] was incubated at 37°C for 1 h with liposomes containing purified and in vitro-synthesized ganglioside S2-6GA2. After incubation, the virions that were not absorbed in the liposomes were isolated, diluted, and reinfected into MDCK cells for three days. Decreases in TCID50 value were investigated for different ganglioside concentrations (0 to 150 μg). Synthesized ganglioside S2-6GA2 and substrate GA2 were prepared by scraping from the TLC plate after enzyme reaction and extraction with a solvent after enzyme reaction. Each assay was carried out in duplicate.

S2-3PG and S2-6PG in a dose-dependent manner. In contrast, substrate GA2 showed no decrease, indicating that no binding occurred. The ganglioside S2-6GA2 synthesized by #4α2-6ST showed a nearly identical decrease in the TCID50 value as the purified gangliosides. These data clearly show that the in vitro synthesized ganglioside S2-6GA2 has affinity to bind to the influenza virus and with S2-3PG and S2-6PG from mammalian ones.

DISCUSSION

Sialic acids (2-keto-3-deoxynonulosonic acids; Sia) are negatively charged α-keto acids with a nine-carbon backbone. They are commonly found as terminal carbohydrate residues on cell surface glycoconjugates (glycoproteins and GSLs) of higher animals. As the terminal carbohydrate residue, Sia is one of the first molecules encountered in cellular interactions and has been found to play important roles in cellular recognition and communication, as briefly described in the literature (6, 35). There are five linkage patterns: NeuAcα2-3Gal, NeuAcα2-6Gal, NeuAcα2-6GlcNAc, NeuAcα2-6GalNAc, and NeuAcα2-8NeuAc in mammalian glycoconjugates, and these linkages are formed by specific STs. The enzymes that transfer the Sia moiety from CMP-NeuAc to the terminal positions of these key glycoconjugates are known as STs. Besides of Sia formation by STs in mammals, the synthesis of Sia by STs in microbial cells are also reported in lipooligosacharide outer core structures, mainly pathogenic bacteria, and their presence is often associated with virulence (12, 36).

In this study, we synthesized very unique gangliosides in vitro and identified their detailed chemical structures. This is the first example of uncommon ganglioside synthesis using bacterial STs. Unique gangliosides, S2-6GA2 and S2-6GA1, were identified as NeuAcα2-6GalNAcβ1-4Galβ1-4Glcβ-Cer and NeuAcα2-6Galβ1-3GalNAcβ1-4Galβ1-4Glcβ-Cer by several analytical techniques, respectively. This result indicated that α2-6STs from marine bacterial sources were able to transfer NeuAc not only to terminal Gal but also to the terminal GalNAc of acceptor substrate. Identical ganglioside synthesis was also expected in the case of GA2 substrate by α2-3STs, but no gangliosides were synthesized, even after longer incubation periods or excess use of the STs compared with the normal assay conditions (Fig. 1B, lanes 3–5). Although further investigations require using different temperatures and pHs conditions for ST assay, it is hard to explain the above result at present.

We quantitatively compared the ganglioside products using neolacto/lacto and ganglio series by changing the STs concentrations (Fig. 2). Consequently, capability of ganglioside synthesis using the gangliosides series, such as GA1 and GA2, was found to be about half lower than that for lacto/neolacto series. In both, ganglioside products were obtained correspondingly to the added STs concentration up to 0.4 U. As this value shows the used units of ST for sialyllactose synthesis, relative sialyltransferase activities for asialo-series substrates are low. Therefore, it seems likely that these values do not necessarily reflect the real ganglioside synthesis. It might be necessary to know the precise critical micelle concentration of individual substrates to the detergent used in this study and use the above concentration in the assay. However, even if GA1 and GA2 showed slightly lower activity in the ganglioside synthesis, these synthesis levels are very reasonable and satisfactory for preparing unique gangliosides. Actually, in the extended scale-up ST (500 μl) in the assay, 80–120 μM in vitro ganglioside synthesis became possible and was used in the subsequent biological assay. Although it is necessary to establish the individual optimal conditions for ST assay, the method described here is one of the most promising so far for practical ganglioside synthesis.

An influenza pandemic occurs when a new human influenza virus emerges in the world. Influenza A and B viruses infect host cells through the binding of viral hemagglutinins to sialylglycoproteins or sialylGSLs, which serve as receptors on the host cell surface; these viruses prefer Sia-containing sugar chain structures, such as sialyllacto-series type I (Siaα2-3/6Galβ1-4GlcNAcβ1-) and type II (Siaα2-3/6Galβ1-3GlcNAcβ1-). It has been reported that influenza viruses differ in their recognition of two types of *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) and linkages (α2-3 or α2-6) of Sia residues (37, 38). Avian and equine viruses preferentially bind Sia α2,3-galactose, whereas human influenza viruses preferentially bind Sia α2,6-galactose (39).

Since the use of influenza virus neuraminidase (NA) inhibitors, especially oseltamivir, is increasing, the emergence of drug-resistant variants has become a major concern. However, conventional host cell lines, such as MDCK and VERO, are insufficient to evaluate drug-resistant variants. Although Sia α2,6-galactose is also in MDCK cells, the amount of Sia α2,6-galactose may be lower than that of epithelial cells in the human airway. Matrovich et al. showed that MDCK cells

overexpressing the human β-galactoside α2,6-sialyltransferase I (ST6Gal I) gene have the potential to assess the sensitivity of human influenza virus isolates to NA inhibitors (40). Interestingly, the ganglioside NeuAcα2-6GalNAcβ1-4Galβ1-4Glcβ-Cer generated by α2-6ST from marine bacterial sources showed a nearly identical decrease in the TCID50 value to the purified gangliosides. These data clearly show that the in vitro-synthesized ganglioside has affinity to bind to the influenza virus as well as S2-3PG and S2-6PG from mammalian ones (Fig. 7). Although we are unable to explain the reasons for the above findings, establishment of stably transfected cell lines with α2-6ST from marine bacteria can be one of the alternative methods to increase influenza virus sensitivities to NA inhibitors and improve them by purposefully changing the concentration of virus receptors on the cell surface based on the unique features of STs from marine bacteria. It will be necessary to further investigate the relationship between the sugar-chain structures and biological functions, such as the preferential binding specificity of the influenza virus, using other variants.

It is reported that the Sialyl-Tn (STn) antigen (NeuAcα2-6GalNAc-Ser/Thr) is enhanced in a wide range of epithelial cancers and is associated with morphological changes, cell growth, and adhesion rates (41, 42). Although STn antigen seems to be related to one of invasive behavior of the cancers, the involved mechanisms remain unclear. Synthesizing the key glycoconjugates, such as Sialyl-Tn (STn) antigen (NeuAcα2-6GalNAc-Ser/Thr) and including the developmental methods introduced here will lead to an efficient method of synthesizing gangliosides for various applications and for investigation of unknown features in the near future (33, 43).

By a series of experiments using various glycolipid substrates, we have shown that not only lacto-series gangliosides [IV^3αNeuAc-nLc_4Cer (S2-3PG) and IV^6αNeuAc-nLc_4Cer; SPG (S2-6PG)] but also other gangliosides (GM1b, NeuAcα2-6GalNAcβ1-4Galβ1-4Glcβ-Cer, and NeuAcα2-6Galβ1-3GalNAcβ1-Galβ1-4Glcβ-Cer) were efficiently synthesized by STs in a large-scale assay, and we confirmed individual linkage specificities by SI-MS analysis after TLC blotting, with methylation studies, and by using monoclonal antibodies. From the all data taken above and including the previous study by us, utilization of these STs from marine bacteria sources have been shown to be a new possibility for large preparations of biologically significant gangliosides in the future. In a further developmental study, we are preparing transformed mammalian expression systems using STs gene from marine bacteria.

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