J. Biochem. 120, 104-110 (1996)

Purification and Characterization of a Marine Bacterial β-Galactoside α2,6-Sialyltransferase from *Photobacterium damsela* JT0160

Takeshi Yamamoto,*[,1] Motoko Nakashizuka,* Hisashi Kodama,† Yasuhiro Kajihara,† and Ichiro Terada*

**Sea Water Science Research Laboratory, Japan Tobacco Inc., 4-13-20 Sakawa, Odawara, Kanagawa 256; and †Life Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227*

Received for publication, February 6, 1996

A bacterial sialyltransferase, named sialyltransferase 0160, was purified from a marine bacterium that had been isolated from seawater from Sagami Bay, Kanagawa. This strain has been identified as *Photobacterium damsela*, and named *P. damsela* JT0160. Sialyltransferase 0160 was purified 688-fold to homogeneity from the crude extract of the cells with a yield of 19% using a combination of anion exchange chromatography, hydroxyapatite chromatography, gel filtration chromatography, and affinity chromatography. The purified enzyme migrated as a single band (61 kDa) on sodium dodecyl sulfate-polyacrylamide gel. This sialyltransferase was found to be a β-galactoside α2,6-sialyltransferase [EC 2.4.99.1] which catalyzes the incorporation of NeuAc from CMP-NeuAc into the galactose residue of the carbohydrate chain at position 6 on the basis of an analysis of the enzymatic reaction products with HPLC, ^{1}H-, ^{13}C-NMR spectroscopy, and fast atom bombardment mass spectroscopy.

Key words: bacterial sialyltransferase, β-galactoside α2,6-sialyltransferase, *Photobacterium damsela*.

Sialic acids play important roles in a variety of biological and physiological events, such as cell-cell recognition and virus infections (*1-4*). Sialic acids exist in a variety of mammalian glycoproteins and glycolipids, and usually occur at the terminal positions of carbohydrate chains of glycoconjugates (*5*). Three linkage patterns, NeuAcα2-6Gal, NeuAcα2-3Gal, and NeuAcα2-6GalNAc are commonly found in mammalian glycoproteins. These linkages are formed by specific sialyltransferases, several of which have been purified from mammalian sources (*6-9*). Each enzyme, which usually catalyzes the transfer of sialic acid from CMP-NeuAc, can be distinguished enzymatically by its rigid specificity for the sequence of the acceptor oligosaccharide and the linkage formed between the sialic acid and the sugar to which it is attached. Sialyltransferases that elaborate the sequences NeuAcα2-6Galβ1-4GlcNAc and NeuAcα2-3Galβ1-4GlcNAc have been purified to homogeneity from bovine colostrum and rat liver. The cloning of the genes of these enzymes, revealed a conserved motif, the sialyl motif, in the catalytic domain of these enzymes (*10*). The presence of this sialyl motif has been used to aid cloning of this gene family, the sialyltransferase family. Similar linkages, NeuAcα2-6Gal and NeuAcα2-3Gal, were also found in the capsular polysaccharides of bacteria, *Vibrio cholerae, Campylobacter jejuni*, and Group B *Streptococcus* (*5, 11-13*). However, sialyltransferase has not yet been purified from bacteria.

The successful purification and cloning of sialyltransferases prompted us to apply them to the modification of the carbohydrate chains of glycoproteins and synthesis of sialosides. Because of the mild reaction conditions, pure sialyltransferases are useful tools for *in vivo* sialylation of soluble or membrane-bound glycoproteins. Replacement of natural sialic acid by synthetic analogues is also possible. Several 9-substituted NeuAc analogues could be transferred onto *N*- or *O*-linked carbohydrate chains of glycoproteins by 4 types of purified mammalian sialyltransferase (*14*). It has been shown that the *in vivo* activity of glycoproteins, such as erythropoietin, is significantly correlated with the carbohydrate structure (*15*). This replacement technique might be useful to modify glycoproteins in order to confer additional functions. Many reports have described the advantages of the enzymatic synthesis of sialoside compared to chemical synthesis. Chemical synthesis requires multi-step procedures and the stereo-specific synthesis of α-sialosides is difficult. Enzymatic synthesis is highly position- and anomer-selective and requires no protection/deprotection steps. Although some sialyltransferases are commercially available, their high cost is a disadvantage for large-scale synthesis of sialosides by enzymes.

Here, we report the purification and the characterization of a bacterial β-galactoside α2,6-sialyltransferase which is more abundant and easier to purify than those from mammalian sources. It should be a useful tool for the modification of glycoproteins and for sialoside synthesis.

[1] To whom correspondence should be addressed at the present address: Tobacco Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227. Phone: +81-45-973-5611, Fax: +81-45-973-6781

MATERIALS AND METHODS

Materials—CMP-[4,5,6,7,8,9-^{14}C]-NeuAc was purchased from Du Pont; CMP-NeuAc, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-β-D-mannopyranoside, methyl-α-D-*N*-acetylglucosaminide, and methyl-β-D-*N*-acetylglucosaminide, were obtained from Sigma; lactose was from Kanto Chemicals; *N*-acetyllactosaminide was from Seikagaku; methyl-α-D-*N*-acetylgalactosaminide and methyl-β-D-*N*-acetyllactosaminide were synthesized according to the procedures described by Sarkar and Kabat (*16*) and Kajihara *et al.*, respectively (*17*); all pyridylaminated carbohydrate chains were from Takara. The structures of the pyridylaminated carbohydrate chains described in this paper are shown in Table I together with the carbohydrate chain numbers, which are used as abbreviations in this paper; marine broth 2216 was from Difco Laboratories; the standard proteins for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were from Daiichi Pure Chem.; Clearsol 1 (scintillation cocktail) was from Nacalai Tesque and all other reagents were commercial products of analytical grade. HiLoad 26/10 Q Sepharose HP, HiLoad 26/60 Sephacryl S-200 HR, and Sephadex G-50 (super fine) were from Pharmacia; hydroxyapatite (2×10 cm) was from Koken; CDP-hexanolamine agarose was from Genzyme; Dowex 1×8 was from Dow Chemical; activated carbon was from Wako Pure Chemical; PALPAK type R was from Takara; AsahiPAK GS-510 was from Asahi Chemical; Sep pak C_{18} was from Millipore; radioactivity was measured using Packard model TR 1900 liquid scintillation counter; ^{1}H- and ^{13}C-NMR spectra were obtained using Bruker AM500 and Bruker AC300 spectrometers, respectively; fast atom bombardment (FAB)-mass spectroscopy was obtained using Kratos Concept IIH.

Isolation and Cultivation of P. damsela JT0160—*P. damsela* JT0160 was isolated from seawater of Sagami bay, Kanagawa.

Marine broth 2216 was used as the culture medium (pH 7.6). The pre-culture was prepared as follows: one colony of *P. damsela* JT0160 on a Marine broth 2216-agar (1.5%) plate was inoculated into 10 ml of Marine broth 2216 medium in a 30 ml test tube and cultivated at 30°C for 8 h on a rotary shaker (150 rpm). This seed culture (5 ml) was transferred to 500 ml of Marine broth 2216 medium in a 3 liter flask. After this, the inoculated seed culture, *P. damsela* JT0160, was cultivated by shaking (150 rpm) at 30°C for 16 h.

Standard Sialyltransferase Assay—Sialyltransferase activity was routinely assayed by measuring [4,5,6,7,8,9-^{14}C]-NeuAc transferred from CMP-[4,5,6,7,8,9-^{14}C]-NeuAc as a donor substrate to lactose as an acceptor substrate. The standard reaction mixture consisted of 70 nmol of CMP-[4,5,6,7,8,9-^{14}C]-NeuAc (642 cpm/nmol), 1.25 μmol of lactose, and enzyme solution in 25 μl of 20 mM sodium cacodylate buffer (pH 5.0) containing 0.02% Triton X-100. The enzyme reaction was carried out at 30°C for 3 min. All assays were performed in duplicate. After the reaction, the reaction mixture was diluted with 5 mM sodium phosphate buffer (pH 6.8) to 2 ml, and applied to a column (0.5×2 cm) of Dowex 1×8 (phosphate form). The eluate (2 ml) was collected directly into a scintillation vial for counting. The radioactivity of [4,5,6,7,8,9-^{14}C]-NeuAc which had transferred to the acceptor substrate in the eluate was measured with a liquid scintillation counter, and the amount of NeuAc transferred was calculated. One unit (U) was defined as the amount of enzyme that transferred 1 μmol of sialic acid per min to the lactose under the conditions described above.

Protein Determination—Protein was determined by the procedure of Lowry *et al.* (*18*) using bovine serum albumin as the standard.

SDS-PAGE—The homogeneity of the purified enzyme was examined by SDS-PAGE on a 12.5% gel by the method described by Laemmli (*19*).

Purification of Sialyltransferase 0160—Each of the following steps was performed at 4°C.

Step 1. Preparation of the crude extract: P. damsela JT0160 cells were harvested from the culture by centrifugation (6,000×*g*, 20 min). The cells were suspended in 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100 and 1 M NaCl, and were sonicated until the absorbance at 660 nm reached 30% of that of the cell suspension. The sonicated solution was centrifuged (100,500×*g*, 60 min) and the supernatant was dialyzed, using cellulose tubing, against 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100. After

TABLE I. **The structures of pyridylaminated carbohydrate chains.**

Abbreviation name	Structure
PA1	Galβ1-4Glc-PA
PA2	NeuAcα2-3Galβ1-4Glc-PA
PA3	Galβ1-4GlcNAcβ1-2Manα1 (6,3) Galβ1-4GlcNAcβ1-2Manα1 → Manβ1-4GlcNAcβ1-4GlcNAc-PA
PA4	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1 (6,3) Galβ1-4GlcNAcβ1-2Manα1 → Manβ1-4GlcNAcβ1-4GlcNAc-PA
PA5	Galβ1-4GlcNAcβ1-2Manα1 (6,3) NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1 → Manβ1-4GlcNAcβ1-4GlcNAc-PA
PA6	NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1 (6,3) NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1 → Manβ1-4GlcNAcβ1-4GlcNAc-PA

dialysis, the precipitate was removed by centrifugation (100,500×*g*, 60 min) to obtain a clarified extract.

Step 2. Q-Sepharose column chromatography: A column of HiLoad 26/10 Q Sepharose HP (2.6×10 cm) was equilibrated with 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100. The clarified extract (80 ml) was applied to the column. The column was washed with 150 ml of the same buffer. The enzyme fraction was eluted with a linear gradient from 0 to 1 M NaCl in the buffer (total elution volume, 1,060 ml). The fractions exhibiting sialyltransferase activity were collected. Desalting of the active fractions was performed by dialysis using cellulose tubing against 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100.

Step 3. Hydroxyapatite column chromatography: A column of hydroxyapatite (2×10 cm) was prepared by equilibrating it with 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100. After the application of the enzyme solution from step 2 (20 ml), the column was washed with 90 ml of the same buffer. The enzyme fraction was eluted with a gradient of potassium phosphate from 0 to 0.35 M (total elution volume, 620 ml). The fractions exhibiting sialyltransferase activity were collected and concentrated by ultrafiltration using Molecut L (Millipore, exclusion molecular mass 10 kDa).

Step 4. Gel-filtration column chromatography: A column of HiLoad 26/60 Sephacryl S-200 HR (2.6×60 cm) was prepared by equilibrating it with 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100 and 0.1 M NaCl. The enzyme solution from step 3 (10 ml) was applied to the column and eluted with the same buffer. The fractions exhibiting sialyltransferase activity were collected. Desalting of the active fractions was performed by dialysis in cellulose tubing against 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100.

Step 5. CDP-hexanolamine-agarose column chromatography: A column of CDP-hexanolamine-agarose (1×3 cm) was prepared by equilibrating it with 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100. The enzyme solution from step 4 (4 ml) was applied to the column. The column was washed with 8 ml of the same buffer. The enzyme fraction was eluted with 6 ml of 2 M NaCl. All the eluate fractions exhibiting sialyltransferase activity were collected.

Analysis of pH and Temperature Profile—To analyze the pH profile for the activity of sialyltransferase 0160, the enzyme reaction was carried out at 30°C for 3 min according to the standard sialyltransferase assay used in this work, except that 20 mM sodium cacodylate buffer (pH 5.0) was replaced with 50 mM sodium acetate buffer (pH 3.7 to 5.0), 50 mM sodium cacodylate buffer (pH 5.0 to 7.3), or 50 mM Tris-HCl buffer (pH 7.3 to 8.0). To analyze the temperature profile of the sialyltransferase 0160 activity, the enzyme reaction was carried out at 20, 25, 30, 35, 40, 45, and 50°C for 3 min, according to our standard sialyltransferase assay.

Analysis of Acceptor Substrate Specificity of Sialyltransferase 0160—Specificity for monosaccharides or oligosaccharides: To determine the acceptor substrate specificity of sialyltransferase 0160, the reaction was carried out with a monosaccharide or an oligosaccharide as an acceptor substrate. The monosaccharides or oligosaccharides which were utilized are as follows: methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, methyl-α-D-mannopyranoside, methyl-β-D-mannopyranoside, methyl-α-D-*N*-acetylglucosaminide, methyl-β-D-*N*-acetylglucosaminide, methyl-α-D-*N*-acetylgalactosaminide, lactose, and *N*-acetyllactosaminide. Each reaction mixture had the following components: monosaccharide or oligosaccharide (0.5 μmol), CMP-[4,5,6,7,8,9-^{14}C]-NeuAc (642 cpm/nmol, 70 nmol), and purified sialyltransferase 0160 (4 mU) in 25 μl of 20 mM sodium cacodylate buffer (pH 5.0) containing 0.02% Triton X-100. The reaction was carried out at 30°C for 3 min. After the reaction, the mixture was diluted with 5 mM sodium phosphate buffer (pH 6.8) to 2 ml, and applied to a column (0.5×2 cm) of Dowex 1×8 (phosphate form). The eluate (2 ml) was collected directly into a scintillation vial for counting. The radioactivity of [4,5,6,7,8,9-^{14}C]-NeuAc which had been transferred to the acceptor substrate in the eluate was measured with a liquid scintillation counter, and the amount of NeuAc transferred was calculated.

Kinetic studies: Apparent kinetic parameters of sialyltransferase 0160 for the donor substrate CMP-NeuAc were determined under the following conditions using lactose as an acceptor substrate. The reaction mixture was composed of lactose (1.25 μmol), various concentrations of CMP-[4,5,6,7,8,9-^{14}C]-NeuAc and purified sialyltransferase 0160 (3 mU) in 25 μl of 20 mM sodium cacodylate buffer (pH 5.0) containing 0.02% Triton X-100. The enzyme reaction was carried out at 30°C for 3 min. Assays were performed in duplicate. Apparent kinetic parameters of sialyltransferase 0160 for acceptor substrates, methyl-β-D-galactopyranoside, lactose and *N*-acetyllactosaminide, were determined with a saturating concentration of CMP-[4,5,6,7,8,9-^{14}C]-NeuAc. The reaction mixture was composed of CMP-[4,5,6,7,8,9-^{14}C]-NeuAc (642 cpm/nmol, 70 nmol), various concentrations of acceptor substrate, and purified sialyltransferase 0160 (3 mU) in 25 μl of 20 mM sodium cacodylate buffer (pH 5.0) containing 0.02% Triton X-100. The enzyme reaction was carried out at 30°C for 3 min. Assays were performed in duplicate. After the reaction, the radioactive product was isolated as described in the "Standard sialyltransferase assay" section, and the enzyme activity was calculated. The kinetic parameters, K_m and V_{max}, were determined from a Lineweaver-Burk plot.

Identification of Enzymatic Reaction Products—Method 1. Product identification by HPLC: To identify sialyloligosaccharide products by HPLC, each enzymatic reaction was carried out with a pyridylaminated carbohydrate chain as an acceptor substrate. Pyridylaminated carbohydrate chains used were PA1, PA3, PA4, and PA5. The reaction mixture was composed of CMP-NeuAc (143 pmol), a pyridylaminated carbohydrate chain (50 pmol), and purified sialyltransferase 0160 (3 mU) in 25 μl of 20 mM sodium cacodylate buffer (pH 5.0) containing 0.02% Triton X-100. The reaction was done at 30°C for 20 h. After the reaction, each mixture was analyzed by HPLC using a Takara PALPAK type R (0.46×25 cm) analytical column. The reaction mixture was applied to the column equilibrated with 100 mM acetic acid-triethylamine buffer (pH 5.2) containing 0.15% *n*-butanol. Pyridylaminated carbohydrate chains (non-reacted acceptor substrate and product) were eluted using *n*-butanol in the same buffer. The concentration of *n*-butanol was increased linearly from

0.15 to 0.5% (0-35 min). Pyridylaminated carbohydrate chains were detected by fluorescence (Ex: 320 nm; Em: 400 nm). The column temperature was 40°C, and the flow rate was 1 ml/min (*20*).

Method 2. Product identification by ^{1}H-, ^{13}C-NMR spectroscopy, and FAB-mass spectroscopy: To identify the enzymatic reaction products, the enzymatic reaction was carried out using methyl-β-D-*N*-acetyllactosaminide (Galβ1-4GlcNAcβ-Me) as an acceptor substrate, and the product was analyzed by ^{1}H-, ^{13}C-NMR spectroscopy, and FAB-mass spectroscopy. The reaction mixture was composed of methyl-β-D-*N*-acetyllactosaminide (200 μmol, 79.4 mg), CMP-NeuAc (16 μmol, 9.6 mg), and 2.5 U of purified sialyltransferase 0160 in 1 ml of 20 mM sodium cacodylate buffer (pH 5.0) containing 0.02% Triton X-100. The reaction mixture was incubated at 30°C for 1 h. The product was purified according to the method of Sabesan and Paulson (*21*), as follows. After the reaction, the reaction mixture was diluted with distilled water to 10 ml and applied to a column (1.5×9 cm) of Dowex 1×8 (phosphate form, 200-400 mesh). The column was washed with distilled water, and then the product was eluted with 5 mM sodium phosphate buffer (pH 6.8; total volume 60 ml). The fractions containing glycosidic NeuAc (determined by the periodate-resorcinol assay) were pooled and evaporated to afford a residue (*22*). The dried residue was dissolved in 2 ml of distilled water, and then applied to a column (1.5× 6.8 cm) of activated carbon. The column was washed with 25 ml of distilled water. The product was eluted with 25 ml each of 10, 20, 50, and 100% ethanol, respectively. The fractions containing glycosidic NeuAc (determined by the periodate-resorcinol assay) were pooled and evaporated to afford a residue. The product was analyzed by ^{1}H-, ^{13}C-NMR spectroscopy, and FAB-mass spectroscopy.

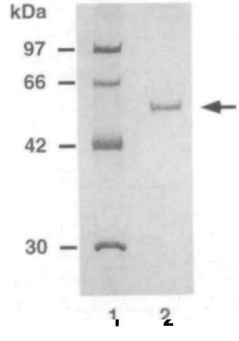

Fig. 1. **SDS-PAGE of purified sialyltransferase 0160.** The electrophoresis was carried out on a 12.5% polyacrylamide gel at pH 8.4 in Tris-glycine buffer. Proteins were located by silver staining. Phosphorylase (97 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), and carbonic anhydrase (30 kDa) were used as standards for molecular mass determination. Lane 1, standard proteins: molecular masses are indicated; lane 2, sialyltransferase 0160.

RESULTS

Purification of Sialyltransferase 0160—Sialyltransferase 0160 was purified from the cells of *P. damsela* JT0160 by the procedures described in "MATERIALS AND METHODS." The purification of sialyltransferase 0160 is summarized in Table II. The enzyme was purified 688-fold, with a yield of 19%. The purified enzyme migrated as a single polypeptide with a molecular mass of 61 kDa under denaturing conditions (Fig. 1). The native protein was estimated to have a molecular mass of 64 kDa by HPLC with an AsahiPAK GS-510 gel filtration column (data not shown). These data indicate that this enzyme is composed of a single polypeptide chain.

General Properties of Sialyltransferase 0160—The isoelectric point of sialyltransferase 0160 was determined to be 4.6 by isoelectrophoresis. The optimum temperature for the activity of sialyltransferase 0160 was found to be 30°C. After having been heated to 40°C for 5 min, this enzyme retained more than 80% of the activity measured at 30°C for 5 min, but the remaining activity fell to less than 5% after treatment at 55°C for 5 min. The optimum pH for the activity of sialyltransferase 0160 was found to be 5. Sialyltransferase 0160 was stable in 20 mM sodium cacodylate buffer (pH 5.0) without glycerol for at least 3 months at −80°C.

Specificity of Sialyltransferase 0160—Sialyltransferase 0160 showed donor substrate specificity for CMP-NeuAc, and the K_m value for CMP-NeuAc was 320 μM. Other sugar nucleotides such as UDP-Gal, UDP-GalNAc, and UDP-Glc

TABLE III. **Monosaccharide and oligosaccharide acceptor specificities of sialyltransferase.** Each reaction mixture (25 μl) contained 0.5 μl of sodium cacodylate, 0.5 μmol of acceptor substrate, 70 nmol of CMP-NeuAc (642 cpm/nmol), 4 mU of sialyltransferase 0160, and 0.02% Triton X-100. The reaction conditions were described under "MATERIALS AND METHODS."

Acceptor (20 mM)	NeuAc transferred (nmol/min)
Methyl-α-D-galactopyranoside	0.12
Methyl-β-D-galactopyranoside	0.35
Methyl-α-D-glucopyranoside	0.02
Methyl-β-D-glucopyranoside	0.03
Methyl-α-D-mannopyranoside	0.02
Methyl-β-D-mannopyranoside	0.03
Methyl-α-D-*N*-acetylgalactosaminide	0.11
Methyl-α-D-*N*-acetylglucosaminide	0.01
Methyl-β-D-*N*-acetylglucosaminide	0.02
Lactose	3.80
N-Acetyllactosaminide	2.37

TABLE II. **Purification of sialyltransferase 0160.**

Step	Volume (ml)	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	760	2,584	21.1	0.008	100	1
Q-Sepharose	240	552	12.4	0.022	59	2.8
Hydroxyapatite	120	85	8.0	0.094	38	11.8
Sephacryl S-200	30	20.1	6.7	0.3	32	37.5
CDP-hexanolamine-agarose	15	0.75	4.1	5.5	19	687.5

[a]One unit (U) was defined as the amount of enzyme that transferred 1 μmol of NeuAc per min to lactose as described under "MATERIALS AND METHODS."

TABLE IV. **Kinetic parameters of sialyltransferase 0160 for donor and acceptor substrate.** These kinetic parameters were determined by the method described under "MATERIALS AND METHODS."

Compound	K_m (mM)	V_{max} (units/mg)	V_{max}/K_m
Donor substrate			
CMP-NeuAc	0.32	7.7	24.0
Acceptor substrate			
Lactose	6.82	6.4	0.93
N-Acetyllactosaminide	8.95	9.7	1.08
Methyl-β-D-galactopyranoside	174	6.4	0.04

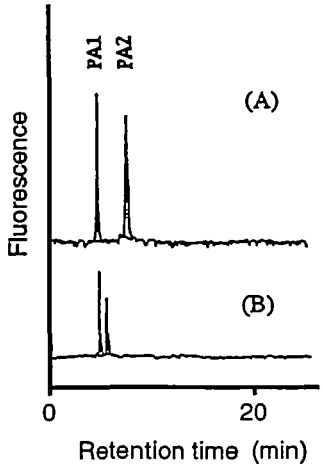

Fig. 2. **HPLC profiles of the enzymatic reaction product using PA1 as an acceptor substrate.** Analysis conditions are described under the experimental procedure. (A) PA1 and PA2, (B) the enzymatic reaction product using PA1.

did not act as donor substrates. Using various monosaccharides or oligosaccharides as acceptor substrates, the acceptor substrate specificity was analyzed. These results are summarized in Table III. The best acceptor substrate was methyl-β-D-galactopyranoside among these monosaccharides. Sialyltransferase 0160 showed anomer selectivity, and the β-anomer was a better acceptor substrate than the α-anomer. The K_m value for *N*-acetyllactosaminide was the same as that of lactose. The K_m value for methyl-β-D-galactopyranoside was about 25 times higher than that of lactose (Table IV).

Product Identification by HPLC—The enzymatic reaction was carried out with pyridylaminated carbohydrate chain as an acceptor substrate, and the reaction products were analyzed by HPLC. The retention time of PA1 (pyridylaminated lactose) was 4.8 min and that of the reaction product from PA1 was 5.6 min. The retention time of PA2 (pyridylaminated 3′-sialyllactose) was 7.6 min (Fig. 2). These results indicate that sialyltransferase 0160 is not a β-galactoside α2,3-sialyltransferase. Furthermore, the retention times of PA3, PA4, PA5, and PA6 were 26.7, 29.4, 28.0, and 31.7 min, respectively. The retention times of all the main reaction products from PA3, PA4, and PA5 as acceptor substrates were 31.7 min (Fig. 3). These results indicate that sialyltransferase 0160 transferred NeuAc from CMP-NeuAc to the galactose residue of these carbohydrate chains at position 6.

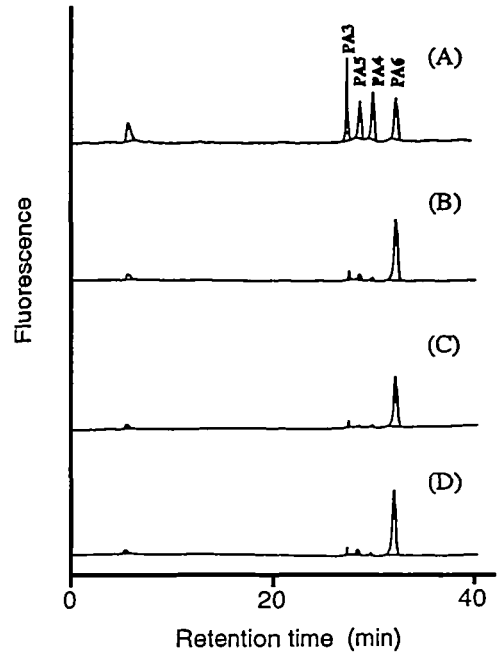

Fig. 3. **HPLC profiles of the enzyme reaction products using PA3, PA4, and PA5 as acceptor substrates.** Analysis conditions are described in the text. (A) PA3, PA4, PA5, and PA6, (B) the enzymatic reaction product using PA3, (C) the enzymatic reaction product using PA4, (D) the enzymatic reaction product using PA5.

TABLE V. **Chemical shifts of reaction product and NeuAcα2, 6-LacNAc.**

Sugar unit	Carbon atom	Reaction product	NeuAcα2,6LacNAc[a]
β-D-GlcNAc	1	102.12	102.08
	2	55.13	55.12
	3	72.84	72.83
	4	81.15	81.19
	5	74.89	74.88
	6	60.77	60.79
	N-C=O	175.06	175.30
	CH_3	22.69	22.69
β-D-Gal	1	103.87	103.86
	2	71.13	71.12
	3	72.96	72.95
	4	68.80	68.77
	5	74.08	74.07
	6	63.74	63.72
α-D-NeuAc(2,6)	1	173.90	173.80
	2	100.57	100.54
	3	40.46	40.49
	4	68.59	68.59
	5	52.31	52.30
	6	72.96	72.95
	7	68.59	68.59
	8	72.10	72.09
	9	63.07	63.06
	N-C=O	175.35	175.61
	CH_3	22.43	22.42

[a]These values were presented by Sabesan and Paulson (*21*).

Product Identification by ^1H-, ^{13}C-NMR Spectroscopy, and FAB-Mass Spectroscopy—To identify the enzymatic reaction products by ^1H-, ^{13}C-NMR spectroscopy, and FAB-mass spectroscopy, the enzymatic reaction was carried out

using methyl-β-D-*N*-acetyllactosaminide (Galβ1-4GlcNAcβ-Me) as the acceptor substrate. The products were purified and isolated. Under the conditions described in "MATERIALS AND METHODS," all of the NeuAc from the donor substrate was transferred to methyl-β-D-*N*-acetyllactosaminide, judging from the results of the control experiment using CMP-[4,5,6,7,8,9-^{14}C]-NeuAc as a donor substrate. Sixteen micromoles of sialyl methyl-β-D-*N*-acetyllactosaminide was synthesized. The FAB-mass spectrum of the product showed the $(M+H)^+$ ion peak at 689. This suggests that the product is sialylmethyl-β-D-*N*-acetyllactosaminide. The proton NMR spectrum showed good agreement with that reported for 2,6 sialoside, NeuAcα2-6Galβ1-4GlcNAcβ-Me (*21*). The chemical shifts of ^{13}C-NMR spectrum were identical with those reported (Table V). From these results, sialyltransferase 0160 was identified as a β-galactoside α2,6-sialyltransferase.

DISCUSSION

Two sialic acid-related enzymes, CMP-NeuAc synthetase and polysialic acid synthetase complex, have been cloned from *E. coli* (*23-25*). However, ours is the first report on the purification of sialyltransferase from a bacterium to homogeneity. Sialyltransferase 0160 reported in this paper catalyzed the incorporation of NeuAc from CMP-NeuAc onto a galactose residue with an α2-6 linkage. This type of linkage, commonly distributed in mammals, is also found in the capsular polysaccharide of Group B *Streptococcus*. Like mammalian sialyltransferase, sialyltransferase 0160 might participate in the formation of this linkage.

The kinetic study showed the K_m value for the donor substrate CMP-NeuAc to be 320 μM, which is slightly higher than that of mammalian sialyltransferase. Its acceptor specificity was similar to that of rat liver α2,6-sialyltransferase. The methyl-β-D-galactopyranoside is the best acceptor substrate among the monosaccharides, and methyl-α-D-*N*-acetylgalactosaminide is also a good acceptor. Preference for the β-anomer is observed for these monosaccharide acceptors. Since lactose was a better substrate than methyl-β-D-galactopyranoside, sialyltransferase 0160 recognizes not only the non-reducing terminal galactose unit, but also the reducing terminal unit to which the galactose is linked. However, its recognition of the non-reducing terminus seemed to be less strict than that of rat liver sialyltransferase. In the case of rat liver α2,6-sialyltransferase, the K_m value for the lactose is about 33-fold higher than that of *N*-acetyllactosaminide. On the other hand, in the case of sialyltransferase 0160, lactose has almost the same K_m value as *N*-acetyllactosaminide. These results indicate that sialyltransferase 0160 does not recognize the 2-acetamido group in the *N*-acetylglucosaminyl residue.

Sialyltransferase 0160 shares some properties, from the viewpoint of the purification, with mammalian sialyltransferases. It required a detergent for extraction, and CDP-hexanolamine conjugate column chromatography was effective in the purification. However, an obvious difference is the ease of purification. Sialyltransferase from rat liver needs to be purified more than ten thousand-fold in order to obtain pure material from enzyme extracts. On the other hand, only six hundred-fold purification was necessary to obtain pure sialyltransferase 0160. Sialyltransferase 0160 can be produced on a large scale without difficulty. Under our culture conditions and in our culture medium, 1 mg of pure sialyltransferase 0160 was obtained from 13.5 liters of culture of *Photobacterium damsela* JT0160 by means of a simple purification procedure. It shoud be possible to increase the productivity of sialyltransferase 0160 by examination of the culture conditions and culture medium. An abundant supply of sialyltransferase is necessary for the enzymatic synthesis of sialoside on a large scale. In this respect, bacterial sialyltransferases have great advantages over those from mammals. Sialyltransferase 0160 might be applicable to sialoside synthesis on an industrial scale.

We would like to thank Masayoshi Kusama, Tobacco Science Research Laboratory, Japan Tobacco Inc., for the fast atom bombardment mass spectrometric analysis.

REFERENCES

1. Paulson, J.C. (1989) Glycoproteins: What are the sugar chains for? *Trends Biochem. Sci.* **14**, 272-276
2. Hakomori, S. (1990) Bifunctional role of glycosphingolipids. *J. Biol. Chem.* **265**, 18713-18716
3. Lasky, L.A. (1992) Selectins: Interpreters of cell-specific carbohydrate information during inflammation. *Science* **258**, 964-969
4. Feizi, T. (1985) Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipid are oncodevelopmental antigens. *Nature* **314**, 53-57
5. Schauer, R. (1982) Chemistry, metabolism, and biological functions of sialic acids in *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 40, pp. 131-234, Academic Press, New York
6. Paulson, J.C., Beranek, W.E., and Hill, R.L. (1977) Purification of a sialyltransferase from bovine colostrum by affinity chromatography on CDP-agarose. *J. Biol. Chem.* **252**, 2356-2362
7. Weinstein, J., de Souza-e-Silva, U., and Paulson, J.C. (1982) Purification of a Galβ1,4GlcNAc α2,6 sialyltransferase and a Galβ1,3(4)GlcNAc α2,3 sialyltransferase to homogeneity from rat liver. *J. Biol. Chem.* **257**, 13835-13844
8. Joziasse, D.H., Bergh, M.L. E., ter Hart, H.G. J., Koppen, P.L., Hooghwinkel, G.J.M., and van den Eijnden, D.H. (1985) Purification and enzymatic characterization of CMP-sialic acid: β-Galactosyl-1,3-*N*-acetylgalactosaminide α-2,3-sialyltransferase from human placenta. *J. Biol. Chem.* **260**, 4941-4951
9. Miyagi, T. and Tsuiki, S. (1982) Purification and characterization of β-galactoside(α2,6)sialyltransferase from rat liver and hepatomas. *Eur. J. Biochem.* **126**, 253-261
10. Livingston, B.D. and Paulson, J.C. (1993) Polymerase chain reaction cloning of a developmentally regulated member of the sialyltransferase gene family. *J. Biol. Chem.* **268**, 11504-11507
11. Vinogradov, E.V., Holst, O., Thomas-Oates, J.E., Broady, K.W., and Brade, H. (1992) The structure of the *O*-antigenic polysaccharide from lipopolysaccharide of *Vibrio cholerae* strain H11-(non-O1). *Eur. J. Biochem.* **210**, 491-498
12. Aspinall, G.O., McDonald, A.G., Raju, T.S., Pang, H., Moran, A.P., and Penner, J.L. (1993) Chemical structures of the core regions of *Campylobacter jejuni* serotypes O:1, O:4, O:23, and O:36 lipopolysaccharides. *Eur. J. Biochem.* **213**, 1017-1027
13. Jennings, H.J. (1983) Capsular polysaccharides as human vaccines in *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 41, pp. 155-208, Academic Press, New York
14. Gross, H.J., Rose, U., Krause, J.M., Paulson, J.C., Schmid, K., Feeney, R.E., and Brossmer, R. (1989) Transfer of synthetic sialic acid analogues to N- and O-linked glycoprotein glycans using four different mammalian sialyltransferases. *Biochemistry* **28**, 7386-7392
15. Wasley, L.C., Timony, G., Murtha, P., Stoudemire, J., Dorner, A.J., Caro, J., Krieger, M., and Kaufman, R.J. (1991) The importance of N-linked and O-linked oligosaccharides for the biosynthesis and *in vitro* and *in vivo* biologic activities of erythropoietin. *Blood* **77**, 2624-2632

16. Sarkar, M. and Kabat, E.A. (1979) Synthesis of the anomers of methyl 2-acetamido-2-deoxy-D-galacto-furanoside and -pyranoside. *Carbohydr. Res.* **69**, 143-149
17. Kajihara, Y., Kodama, H., Wakabayashi, T., Sato, K., and Hashimoto, H. (1993) Characterization of inhibitory activities and binding mode of synthetic 6′-modified methyl N-acetyl-β-lactosaminide toward rat liver CMP-D-Neu5Ac:D-galactoside-(2,6)-α-D-sialyltransferase. *Carbohydr. Res.* **247**, 179-193
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
20. Kondo, A., Suzuki, J., Kuraya, N., Hase, S., Kato, I., and Ikenaka, T. (1990) Improved method for fluorescence labeling of sugar chains with sialic acid residues. *Agric. Biol. Chem.* **54**, 2169-2170
21. Sabesan, S. and Paulson, J.C. (1986) Combined chemical and enzymatic synthesis of sialyloligosaccharides and characterization by 500-MHz ^{1}H and ^{13}C NMR spectroscopy. *J. Am. Chem. Soc.* **108**, 2068-2080
22. Jourdian, G.W., Dean, L., and Roseman, S. (1971) The sialic acids. *J. Biol. Chem.* **246**, 430-435
23. Vann, W.F., Silver, R.P., Abeijon, C., Chang, K., Aaronson, W., Sutton, A., Finn, C.W., and Kotsatos, M. (1987) Purification, properties, and genetic location of *Escherichia coli* cytidine 5′-monophosphate N-acetylneuraminic acid synthetase. *J. Biol. Chem.* **262**, 17556-17562
24. Zapata, G., Vann, W.F., Aaronson, W., Lewis, M.S., and Moos, M. (1989) Sequence of the cloned *Escherichia coli* K1 CMP-N-acetyl-neuraminic acid synthetase gene. *J. Biol. Chem.* **264**, 14769-14774
25. Silver, R.P., Vann, W.F., and Aaronson, W. (1984) Genetic and molecular analyses of *Escherichia coli* K1 antigen genes. *J. Bacteriol.* **157**, 568-575