Preparation of GM1 Ganglioside with Sialidase-Producing Marine Bacteria as a Microbial Biocatalyst

YASUFUMI FUKANO AND MAKOTO ITO*

Laboratory of Marine Biochemistry, Faculty of Agriculture, Kyushu University,
Higashi-ku, Fukuoka 812-81, Japan

Received 2 December 1996/Accepted 28 February 1997

This paper describes the preparation of monosialoganglioside GM1 with sialidase-producing marine bacteria as a microbial biocatalyst. A new sialidase-producing bacterium, identified tentatively as *Pseudomonas* sp. strain YF-2, was isolated from seawater by enrichment culture with ganglioside as the sole source of carbon. When YF-2 was cultured in a synthetic medium containing crude bovine brain gangliosides at 25°C for 3 days, 80 to 90% of the gangliosides were converted to GM1. GM1 was then purified from the supernatant of YF-2 culture by C₁₈ reverse-phased chromatography, followed by DEAE-Sephadex A25 anion-exchange chromatography. In a typical experiment, 178 mg of highly purified GM1 was obtained from 500 mg of the crude ganglioside fraction. The GM1 induced neurite outgrowth of neuroblastoma Neuro2a cells at a concentration of 33 to 100 µM in the presence of fetal calf serum. Sialidase was purified 33-fold with 13.3% recovery from the culture supernatant of YF-2. The purified enzyme hydrolyzed polysialogangliosides to produce GM1 but did not act on GM1. It was therefore concluded that polysialogangliosides in the culture of strain YF-2 were converted to GM1 by this sialidase.

Gangliosides, glycosphingolipids containing sialic acids, occur most prominently in cerebral and nervous tissues (16). Gangliosides were found to modulate the function of various membrane proteins including enzymes (19), ion channels (8, 26), receptors (4, 5, 18), and cell adhesion molecules (27). They are divided into several groups according to the structure of the backbone saccharides. The gangliotetraose family is a major ganglioside of mature neurons and is classified, depending on the number of linked sialic acid residues per molecule, into the following: GM1 (having one sialic acid), GD1 (having two sialic acids), GT1 (having three sialic acids), and GQ1 (having four sialic acids). Recently, clinical applications of GM1 for neurological disorders such as Alzheimer's disease (23), Parkinson's disease (21), spinal-cord injury (9), and stroke (2) have been reported.

GM1 is usually prepared from bovine brain gangliosides (12). However, these ganglioside preparations contain less than 30% GM1, the rest being polysialogangliosides which contain two or more sialic acid residues. To prepare GM1 from polysialogangliosides, sialic acids are removed by either a sialidase or an acid treatment. However, both methods are unsuitable for mass production of GM1, since the conversion rate of polysialogangliosides to GM1 is quite low after digestion with any of the commercially available sialidases and the use of HCl or H₂SO₄ also results in the removal of sialic acids from GM1, producing asialo GM1. We thus developed a new method for mass production of GM1 that employs sialidase-producing bacteria as a biocatalyst instead of using isolated enzyme.

Since polysialogangliosides have been shown to be abundantly present in the brains of marine fish (25), sialidase-producing bacteria capable of degrading polysialogangliosides should be present in marine environments. In this study, we tried to isolate the bacteria that produce sialidase from marine environments. This paper describes the isolation and identification

of the sialidase-producing bacterium (YF-2), the method for preparing GM1 by this strain, characteristics of the GM1 prepared (including its ability to induce neurite outgrowth in Neuro2a cells), and finally, characteristics of the sialidase produced by YF-2.

MATERIALS AND METHODS

Materials. Crude gangliosides were prepared from bovine brain as previously described (12). The following gangliosides were purchased from Iatron Laboratories, Inc.: GM3, GM2, GM1a, GD1a, GD1b, GT1b, GD3, and GQ1b. *Vibrio cholerae* sialidase was purchased from Sigma. 4-Methylumbelliferyl- α -D-N-acetylneuraminic acid (4-MU-NeuAc) was purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Precoated Silica Gel 60 thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). Neuroblastoma Neuro2a cells were kindly donated by H. Higashi (Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan).

Identification of strain YF-2. The identification of YF-2 was conducted according to the 8th edition of *Bergey's Manual of Determinative Bacteriology* (6). The incubation temperature was 25°C except for special cases. Motility, morphology, and gram-staining characteristics were determined by light microscopy. A transmitting electron microscope was used for observation of flagella after staining with 2% p-tungstic acid. The oxidase test of Kovacs (14) was used, and the utilization of glucose was examined by the method of Hugh and Leifson (O-F test) (10). Other usual identification methods were employed as required. YF-2 cells were maintained in our laboratory on an agar slant culture containing gangliosides (0.05% crude bovine brain gangliosides, 0.5% polypeptone, 0.1% yeast extract, 2% NaCl, and 1.8% agar, pH 7.4).

Preparation of GM1 with strain YF-2. Cells from agar slant culture were transferred into 5 ml of synthetic medium A (0.05% NH₄Cl, 0.05% K₂HPO₄, 1.0% NaCl, pH 7.4) containing 0.5% crude bovine brain gangliosides as the sole source of carbon and incubated at 25°C for 16 h with shaking. The concentration of cells in this seeded culture was 1.1×10^7 /ml. The seeded culture was transferred into 95 ml of synthetic medium A containing 0.5% gangliosides and incubated at 25°C for 3 days with shaking. The supernatant, obtained by centrifugation at $7,200 \times g$ for 15 min, was applied to a C₁₈ silica column (30-g gel, diameter, 30 mm, Waters) which was equilibrated with methanol. After washing the column with 300 ml of distilled water, fractions including GM1 were eluted with 500 ml of chloroform-methanol (2/1, vol/vol). The eluted glycolipids were dissolved in 50 ml of chloroform-methanol-water (30/60/8, vol/vol) and loaded on a DEAE-Sephadex A25 column (volume, 75 ml; diameter, 25 mm, Pharmacia) which was equilibrated with the same solvent. After being washed with 300 ml of the same solvent, GM1 was eluted from the column by using a linear gradient of 400 ml each of the same solvent and the same solvent containing 0.8 M sodium acetate. Five microliters of each fraction was applied to a TLC plate which was developed with solvent I (chloroform-methanol-0.02% CaCl₂, 5/4/1, vol/vol). The TLC plate was stained with orcinol-H₂SO₄ reagent and quantified with a Shimadzu CS-9300 chromatoscanner with the reflectance mode set at 540 nm.

* Corresponding author. Mailing address: Laboratory of Marine Biochemistry, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-81, Japan. Phone: 81-92-642-2900. Fax: 81-92-642-2900. E-mail: makoto_i@agr.kyushu-u.ac.jp.

FIG. 1. Electron micrograph of *Pseudomonas* sp. strain YF-2. YF-2 cells were stained with 2% *p*-tungstic acid solution (pH 7.2) and observed under an electron microscope (JEM 100 CXII, JEOL). The body length and width of this bacterium are about 0.8 to 1.2 μm and 0.5 to 0.6 μm , respectively.

Purity of the final preparation of GM1 was determined by staining with orcinol- H_2SO_4 , resorcinol-HCl, and Coomassie brilliant blue (17).

Fast-atom bombardment mass spectrometry (FAB-MS) analysis. GM1 was analyzed by FAB-MS (JMS HX-100; JEOL Ltd., Tokyo, Japan) by using the negative ion mode with a triethanolamine as the matrix (3).

Cell culture of neuroblastoma Neuro2a cells and quantification of neurite. Cells (10^5) were cultured in a 6-well plate containing 1 ml of minimum essential medium supplemented with 10% heat-inactivated fetal calf serum (FCS) in an incubator with a humidified mixture of 95% air and 5% CO_2 . GM1 was dissolved in distilled water, sonicated in a sonic bath, and added to the medium. After cultivation of cells with GM1 for 24 h, photographs were taken randomly under a microscope (magnification, $\times 100$). To quantify neuritogenesis, more than 100 cells in each well were counted. Processes extending longer than the diameter of the cell body were considered neurites in this study.

Enzyme assay of sialidase. Sialidase activity was measured by using 4-MU-NeuAc as the substrate during the course of purification of the enzyme. The reaction mixture contained 1 nmol of 4-MU-NeuAc and an appropriate amount of enzyme in 100 μl of 20 mM sodium acetate buffer, pH 5.5. Following incubation at 37°C for 30 min, the reaction was stopped by adding 100 μl of 1 M glycine-NaOH buffer, pH 10.4. Enzyme activity was measured by determination of absorbance at 325 nm. The control experiment was conducted with the heat-inactivated enzyme. One unit of the enzyme was defined as the amount which catalyzes the release of 1 μmol of 4-MU per min from 4-MU-NeuAc under the conditions described above. Sialidase activity was also measured by using various gangliosides as the substrate. The reaction mixture contained 1.25 nmol of gangliosides and an appropriate amount of enzyme in 20 μl of 20 mM sodium acetate buffer, pH 5.5. Following incubation at 37°C for 30 min, the reaction was stopped by boiling at 100°C for 5 min. Each reaction mixture was dried by a Speed Vac concentrator, dissolved in 5 μl of chloroform-methanol (2/1), and applied to a TLC plate which was developed with solvent I. TLC plates were visualized with orcinol- H_2SO_4 staining and quantified with a Shimadzu CS-9300 chromatoscanner with the reflectance mode set at 540 nm. The extent of hydrolysis was calculated as follows: hydrolysis (%) = (peak area for glycolipid produced) $\times$ 100/(peak area for glycolipid produced + peak area for remaining glycolipid).

Purification of sialidase. Inocula from agar slant cultures were introduced into a cotton-plugged 200-ml flask containing 60 ml of sterilized liquid medium (0.5% polypeptone, 0.1% yeast extract, 2.0% NaCl, and 0.1% bovine brain acetone powder, pH 7.4) and incubated at 25°C for 1 day with vigorous shaking. Two aliquots of 30 ml of the culture were then separately transferred to cotton-plugged 5-liter flasks each containing 2.25 liters of the same medium as above and incubated at 25°C for 3 days with vigorous shaking. The culture supernatant (4.08 U, 4.5 liters) obtained was adjusted to 75% saturation with solid ammonium sulfate and allowed to stand overnight at 4°C. The precipitate was collected by centrifugation (11,000 $\times$ g for 60 min) and dissolved in 220 ml of cold distilled

water. After being dialyzed against 20 mM sodium acetate buffer, pH 5.5 (buffer A), the enzyme solution was run on a DEAM column (2.2 by 15 cm; Yamazen Co., Osaka, Japan), which previously had been equilibrated with buffer A, by using a BPLC-600FC high-performance liquid chromatography (HPLC) system

TABLE 1. Morphological, physiological, and biochemical properties of strain YF-2^a

Characteristic	YF-2
Shape	Short rod
Motility	+
Flagellum	Polar monotrichous
Gram staining	—
Growth in air	—
Growth anaerobically	—
Catalase	+
Oxidase	—
O-F test	—
Color of colony	Yellowish white
Na ⁺ requirement	+
Growth in presence of:	
0% NaCl	—
1% NaCl	+
2% NaCl	++
3% NaCl	+
Degradation of DNA	—
Degradation of gelatin	—
Arginine dehydrolase	+
Growth at 4 or 37°C	—
Utility of:	
Glucose	+
Trehalose	—
L-Valine	—
β -Alanine	—
L-Arginine	—
Production of fluorescence pigments	—
Mol% G+C in DNA	68.5

^a ++, Strongly positive; +, positive; —, negative.

FIG. 2. Conversion of polysialogangliosides to GM1 during cultivation with strain YF-2. Five milliliters of YF-2 culture at logarithm phase (1.1×10^7 cells/ml) was transferred to 95 ml of synthetic medium A containing 0.5% (wt/vol) crude bovine brain gangliosides and incubated at 25°C for 3 days with shaking. At the time indicated, 2 μ l of the culture supernatant was withdrawn and the ganglioside pattern was examined by TLC. Data are the percent amounts of each ganglioside relative to total gangliosides (GM1, GD1a, GD1b, and GT1b) at the indicated time points. Details are given in Materials and Methods.

(Yamazen Co.). The column was washed with 6.5 bed volumes of buffer A, and sialidase was then eluted from the column with a linear salt gradient generated by buffer A and buffer A containing 1 M NaCl. The active fractions of sialidase were pooled, concentrated with an Amicon concentrator with a YM10 membrane, and dialyzed against buffer A containing 0.2 M NaCl. The enzyme solution was run on an HW-55F gel filtration column (4.4 by 30 cm, Yamazen Co.) by using a BPLC-600FC HPLC system. The column was equilibrated and eluted with buffer A containing 0.2 M NaCl. The flow rate was 5 ml/min, and fractions of 5 ml were collected. The active fractions were pooled, dialyzed against 2 mM phosphate buffer (pH 7.0), and run on a hydroxyapatite column (2.7 by 18 cm, Yamazen Co.), which previously had been equilibrated with 2 mM phosphate buffer, pH 7.0, by using a BPLC-600FC HPLC system. The column was washed with 2.5 bed volumes of 2 mM phosphate buffer, pH 7.0, and sialidase was then eluted from the column with a linear gradient generated by 2 mM and 200 mM phosphate buffer, pH 7.0. The active fractions of sialidase were pooled, concentrated with an Amicon concentrator with a YM10 membrane, and dialyzed against buffer A. Bovine serum albumin was added to the purified enzyme fractions at a concentration of 1 mg/ml to prevent the loss of enzyme activity.

RESULTS

Isolation and identification of sialidase-producing bacteria.

Sialidase-producing bacteria have been isolated from seawater, sea sand, sea mud, marine algae, and the gills of marine fish by enrichment culture with synthetic medium A containing crude bovine brain gangliosides as the sole source of carbon. This result strongly suggests that sialidase-producing bacteria are widely distributed and could contribute to the degradation of polysialogangliosides in marine environments. It should be noted that polysialogangliosides have been shown to be abundantly present in the brains of marine fish (25). Among the 28 strains isolated as sialidase-producing bacteria, YF-2 was found to have the highest productivity of sialidase acting on gangliosides.

Strain YF-2, isolated from seawater, is a short rod-shaped bacterium with a long polar flagellum (Fig. 1). Optimum growth of YF-2 was observed in medium containing 2% NaCl. Thus, YF-2 is considered to be a typical marine bacterium. The biochemical and physiological properties of YF-2 are summarized in Table 1. From these results, strain YF-2 was tentatively

FIG. 3. Effects of initial concentration of polysialogangliosides on the conversion rate. Two hundred-fifty microliters of YF-2 culture at logarithm phase (1.1×10^7 cells/ml) was transferred to 5 ml of synthetic medium A containing 0.5% (wt/vol) (a), 1% (wt/vol) (b), or 2% (wt/vol) (c) of crude bovine brain gangliosides and incubated at 25°C for 3 days with shaking. Culture supernatants were examined by TLC at the time indicated. Data are the percent amounts of each ganglioside relative to total gangliosides (GM1, GD1a, GD1b and GT1b) at the indicated time points.

FIG. 4. Purity of prepared GM1 analyzed by TLC. Five micrograms of crude bovine brain gangliosides and 3.8 μ g of prepared or standard GM1 were analyzed by TLC with solvent I. (A) Orcinol- H_2SO_4 staining. (B) Resorcinol-HCl staining. (C) Coomassie brilliant blue staining. Lanes 1, crude gangliosides from bovine brain; lanes 2, prepared GM1; lanes 3, standard GM1.

assigned to the genus *Pseudomonas*. This strain was, however, not identical to any species of *Pseudomonas* described in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (4).

Conversion of polysialogangliosides to GM1 by strain YF-2. Five hundred milligrams of crude bovine brain gangliosides was cocultured with strain YF-2 in 100 ml of synthetic medium A at 25°C with shaking. Conversion of polysialogangliosides to GM1 proceeded gradually, and after 3 days the conversion was more than 80% complete (Fig. 2). Asialo GM1 was not detected during the cultivation period, indicating that sialic acids were removed from polysialogangliosides but not from GM1. As shown in Fig. 3, addition of up to 20 mg of crude gangliosides per ml of culture medium (2% wt/vol) did not affect the conversion rate.

Purification and characterization of GM1 from the culture of strain YF-2. Crude gangliosides (500 mg) were cocultured with YF-2 as described in Materials and Methods. After cultivation at 25°C for 3 days, the GM1 was purified from the culture supernatant by C_{18} -silica reversed-phase chromatography, followed by DEAE-Sephadex A25 column chromatography. Finally, 178 mg of GM1 was obtained. The purity of the crude ganglioside preparation used for a starting material was 43%, and thus, the recovery was estimated to be 81%. Figure 4 shows the purity of GM1 obtained from the DEAE-Sephadex column along with crude gangliosides on a TLC plate developed with solvent I after staining with either (i) orcinol- H_2SO_4 (for carbohydrate-containing products), (ii) resorcinol-HCl (for sialic acid-containing products) or (iii) Coomassie brilliant blue (for lipid-containing products). No contamination of the GM1 preparation was observed by TLC, indicating that the GM1 was highly purified. The GM1 was analyzed by FAB-MS by using a negative ion mode. The characteristic pseudomolecular ions $(\text{M}-\text{H})^-$ were found at m/z 1544 and 1572, which corresponded to two molecular species of monosialoganglioside GM1 with a different ceramide. One type of ceramide consists of the sphingosine of d18:1 long-chain base and the fatty acids of C18:0 (M_r , 565) and the other type consists of the eicosasphingosine of d20:1 and the fatty acids of C18:0 (M_r , 593). This result is consistent with the fact that bovine brain GM1 is composed primarily of two types of ceramides (3).

Neurite outgrowth of Neuro2a cells by GM1. GM1 was reported to enhance the neurite extension of neuroblastoma Neuro2a cells in the absence of serum (15). Neurite outgrowth

was observed not only in the absence but also in the presence of serum when the prepared GM1 was added to the culture of Neuro2a cells. The extent of neurite outgrowth was concentration dependent in the presence of 10% FCS (Fig. 5), and the ability of this GM1 to enhance neurite extension was found to be comparable to that of commercially available GM1 (data not shown).

Purification of sialidase. Sialidase was purified 33-fold with 13.3% recovery from a culture supernatant of newly isolated *Pseudomonas* sp. strain YF-2 by anion-exchange, gel filtration and hydroxyapatite chromatographies as described in Materials and Methods. The molecular mass of the sialidase was estimated to be 110 kDa by gel filtration with a TSK-gel G3000SW column.

General properties and specificity of sialidase. The general properties of the enzyme are as follows: optimal activity at pH 5.0 to 5.5 (20 mM sodium acetate buffer) and stable below 35°C; potentially inhibited by Hg^{2+} , Cu^{2+} , and Fe^{2+} at a concentration of 2 mM; and slightly activated by sodium cholate at a concentration of 2 mg/ml when GD1a was used as the substrate. The K_m for GD1a is 0.366 mM. The substrate specificity of the enzyme is summarized in Table 2. The enzyme hydrolyzed GD1a and GM3 most efficiently but did not act on GM1a or GM2. It should be noted that the enzyme also hydrolyzed glycoprotein (fetuin), synthetic substrate (4-MU-NeuAc), and sialyloligosaccharide (sialyl-lactose) (data not shown).

DISCUSSION

It is our experience that polysialogangliosides are quite resistant to commercially available sialidase compared with synthetic substrates or glycoproteins. Removal of sialic acids by chemical methods has been found to produce by-products, mainly asialo GM1. In contrast, the method used with strain YF-2 described here is suitable for the preparation of GM1 in large quantities due to its high yield and low cost. This method has been applied at the laboratory scale (gram level) and could be used to produce GM1 on an industrial scale (kilogram level).

FIG. 5. Effects of concentration of GM1 on neuritogenesis of Neuro2a cells. Neuro2a cells (10^3) were cultured for 24 h in minimum essential medium with 10% FCS in an incubator containing a humidified 95% air-5% CO_2 mixture. The indicated concentration of GM1 were added to the culture at 0 h. The values are the averages from three independent experiments. Error bars indicate standard deviations. Details are given in Materials and Methods.

TABLE 2. Substrate specificity of YF-2 sialidase

Ganglioside	Substrate Structure	Degradation (%) ^a
GM1a ^b	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer	0
GM2	GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer	0
GM3	NeuAcα2-3Galβ1-4Glcβ1-1Cer	55.3
GD1a	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer	43.3
GD1b	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1Cer	27.0
GT1b	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1Cer	27.7

^a Seventeen microunits of the enzyme was incubated at 37°C for 30 min with 1.25 nmol of each substrate in 20 μl of 20 mM sodium acetate buffer, pH 5.5. Degradation was calculated by the method given in Materials and Methods.

^b Abbreviations for gangliosides follow the nomenclature system of Svennerholm (22). In this study, gangliosides having two or more sialic acid residues are referred to as polysialogangliosides.

There have been many reports on sialidase-producing microorganisms, such as *Arthrobacter ureafaciens* (24), *Clostridium perfringens* (7), *Vibrio cholerae* (1), and Newcastle disease virus (20). However, these microorganisms are pathogenic, and their use is not desirable for production of GM1. The marine bacterium YF-2 used for this study was tentatively identified as a *Pseudomonas* sp. but did not correspond to any of the pathogenic species of *Pseudomonas* reported so far.

We confirmed that the conversion of polysialogangliosides to GM1 was entirely due to the action of sialidase produced by YF-2, since YF-2 sialidase can attack both the NeuAcα2,3 linkage bound to terminal galactose and the NeuAcα2,8 linkage bound to NeuAc but not the NeuAcα2,3 linkage directly bound to internal galactose in gangliosides (Table 2). Consequently, polysialogangliosides were converted to GM1, but the GM1 was not converted to asialo GM1. It should be emphasized that no ganglioside-degrading enzymes other than sialidase were detected in the culture of YF-2.

This paper describes an innovative method for mass production of GM1 by using living cells of strain YF-2 instead of isolated enzymes. The concept could also be applied to obtain various (glyco)sphingolipid metabolites by using other (glyco)sphingolipid-degrading microorganisms. The method has been successfully applied to novel (glyco)sphingolipid-degrading bacteria which produce enzymes capable of cleaving the *N*-acyl linkage of ceramides in various glycosphingolipids and sphingomyelin to produce their lyso-forms. This enzyme was tentatively named sphingolipid ceramide *N*-deacylase (SCDase) (11). By using the SCDase-producing bacterium *Pseudomonas* sp. strain TK-4 as the biocatalyst, we succeeded in preparing various lysoglycosphingolipids in large quantities with high yield (13).

ACKNOWLEDGMENTS

We thank S. Yoshida for the FAB-MS analysis of GM1. We also appreciate the help of N. Furuya for observation of bacteria by electron microscopy.

This work was supported in part by a Grant-in Aid for Scientific Research (A) (08556015) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Ada, G. L., E. L. French, and P. E. Lind. 1961. Purification and properties of neuraminidase from *Vibrio cholerae*. *J. Gen. Microbiol.* **24**:409–421.
2. Argentino, C., M. L. Sacchetti, D. Toni, G. Savoini, E. D'Arcangelo, F. Erminio, F. Federico, F. F. Milone, V. Gallai, D. Gambi, A. Mamoli, G. A. Ottonello, O. Ponari, G. Rebucci, U. Senin, and C. Fieschi. 1989. GM1 ganglioside therapy in acute ischemic stroke. *Stroke* **20**:1143–1149.
3. Arita, M., M. Iwamori, T. Higuchi, and Y. Nagai. 1983. Negative ion fast atom bombardment mass spectrometry of gangliosides and asialo gangliosides: a useful method for the structural elucidation of gangliosides and related neutral glycosphingolipids. *J. Biochem.* **94**:249–256.
4. Bremer, E. G., S. Hakomori, D. F. Bowen-Pope, E. Raines, and R. Ross. 1984. Ganglioside-mediated modulation of cell growth, growth factor binding, and receptor phosphorylation. *J. Biol. Chem.* **259**:6818–6825.
5. Bremer, E. G., J. Schlessinger, and S. Hakomori. 1986. Ganglioside-mediated modulation of cell growth. *J. Biol. Chem.* **261**:2434–2440.
6. Buchanan, R. E., and N. E. Gibbons (ed.). 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore, Md.
7. Cassidy, J. T., G. W. Jourdain, and S. Roseman. 1965. The sialic acids. Purification and properties of sialidase from *Clostridium perfringens*. *J. Biol. Chem.* **240**:3501–3506.
8. Frieder, B., and M. M. Rapport. 1987. The effect of antibodies to gangliosides on Ca²⁺ channel-linked release of γ-aminobutyric acid in rat brain slices. *J. Neurochem.* **48**:1048–1052.
9. Geisler, F. H., F. C. Dorsey, and W. P. Coleman. 1991. Recovery of motor function after spinal-cord injury. A randomized, placebo-controlled trial with GM1 ganglioside. *N. Engl. J. Med.* **324**:1829–1838.
10. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. *J. Bacteriol.* **66**:24–26.
11. Ito, M., T. Kurita, and K. Kita. 1995. A novel enzyme that cleaves the *N*-acyl linkage of ceramides in various glycosphingolipids as well as sphingomyelin to produce their lyso forms. *J. Biol. Chem.* **270**:24370–24374.
12. Kanfer, J. N. 1969. Preparation of gangliosides. *Methods Enzymol.* **14**:660–664.
13. Kita, K., S. Mitsutake, and M. Ito. Unpublished data.
14. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature (London)* **178**:703.
15. Ledeen, R. W. 1984. Biology of gangliosides: neuritogenic and neuronotrophic properties. *J. Neurosci. Res.* **12**:147–159.
16. Ledeen, R. W. 1989. Biosynthesis, metabolism, and biological effects of gangliosides, p. 43–83. In R. U. Margolis and R. K. Margolis (ed.), *Neurobiology of glycoconjugates*. Plenum Press, New York, N.Y.
17. Nakamura, K., and S. Handa. 1984. Coomassie brilliant blue staining of lipids on thin-layer plates. *Anal. Biochem.* **142**:406–410.
18. Nojiri, H., M. Stroud, and S. Hakomori. 1991. A specific type of ganglioside as a modulator of insulin-dependent cell growth and insulin receptor tyrosine kinase activity. *J. Biol. Chem.* **266**:4531–4537.
19. Partington, C. R., and J. W. Daly. 1979. Effect of gangliosides on adenylate cyclase activity in rat cerebral cortical membranes. *Mol. Pharmacol.* **15**:484–491.
20. Paulson, J. C., J. Weinstein, L. Dorland, H. van Halbeek, and J. F. G. Vliegenthart. 1982. Newcastle disease virus contains a linkage-specific glycoprotein sialidase. *J. Biol. Chem.* **257**:12734–12738.
21. Schneider, J. S., D. P. Roeltgen, D. S. Rothblat, J. Chapas-Crilly, L. Seraydarian, and J. Rao. 1995. GM1 ganglioside treatment of Parkinson's disease: an open pilot study of safety and efficacy. *Neurology* **45**:1149–1154.
22. Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**:613–623.
23. Svennerholm, L. 1994. Gangliosides. A new therapeutic agent against stroke and Alzheimer's disease. *Life Sci.* **55**:2125–2134.
24. Uchida, Y., Y. Tsukada, and T. Sugimori. 1979. Enzymatic properties of neuraminidases from *Arthrobacter ureafaciens*. *J. Biochem.* **86**:1573–1585.
25. Wakai, H., A. Murata, K. Kon, K. Maruyama, S. Kimura, H. Ogura, and S. Ando. 1993. Isolation and characterization of a trisialyllactosylceramide, GT3, containing an O-acetylated sialic acid in cod fish brain. *J. Biochem.* **113**:502–507.
26. Wu, G., and R. W. Ledeen. 1991. Stimulation of neurite outgrowth in neuroblastoma cells by neuraminidase: putative role of GM1 ganglioside in differentiation. *J. Neurochem.* **56**:95–104.
27. Zheng, M., H. Fang, T. Tsuruoka, T. Tuji, T. Sasaki, and S. Hakomori. 1993. Regulatory role of GM3 ganglioside in α5β1 integrin receptor for fibronectin-mediated adhesion of FUA169 cells. *J. Biol. Chem.* **268**:2217–2222.