J. Biochem. **142,** 403–412 (2007)
doi:10.1093/jb/mvm147

Molecular Cloning, Expression and Properties of an α/β-Galactoside α2,3-Sialyltransferase from *Vibrio* sp. JT-FAJ-16

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Received June 14, 2007; accepted July 3, 2007; published online August 2, 2007

We cloned, expressed and characterized a novel α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. bacterium JT-FAJ-16. Using a α2,3-sialyltransferase gene from a marine bacterium as a probe, a DNA sequence encoding a 402-amino-acid protein was identified from the JT-FAJ-16 genomic library. The protein showed 27.3–64.7% identity to the bacterial sialyltransferases classified into glycosyltransferase family 80. The protein showed sialyltransferase activity when expressed in *Escherichia coli*. The N-terminal truncated form of the enzyme was amplified in *E. coli* and its recovered activity was 215.7 unit/l culture medium. It was purified as a single band on SDS–PAGE through the three chromatographic steps. The specific activity of the purified recombinant enzyme reached 57.5 unit/mg protein. The α2,3sialylation was confirmed by ^{1}H- and ^{13}C-NMR analyses of the reaction products. The enzyme was optimally active at pH 5.5 and at 20°C. Interestingly, the enzyme used both the α- and β-anomers of galactosides as acceptors, suggesting that it can be described as an α/β-galactoside α2,3-sialyltransferase. The enzyme had a wide range of acceptor substrate specificities. It transferred *N*-acetylneuraminic acid (NeuAc) to various monosaccharides and various oligosaccharides, and both *N*-linked and *O*-linked asialo-glycoprotein. These results suggest that the enzyme can be used as a powerful tool for the study for glycotechnology.

Key words: cloning, expression, sialic acids, sialyltransferase, *Vibrio*.

Abbreviations: CMP-NeuAc, cytidine-5′-monophospho-*N*-acetylneuraminic acid; ESIMS, electrospray ionization mass spectrometry; Fuc, fucose; Gal, galactose; Glc, glucose; IPTG, isopropyl-1-thio-β-D-galactopyranoside; [M-H]-, deprotonated molecular ion; MALDI-TOFMS, matrix-assisted laser ionization time-of-flight mass spectrometry; Man, mannose; Me, methyl; NAc, *N*-acetyl; NeuAc, *N*-acetylneuraminic acid; ORF, open reading frame; PA, pyridylaminated; TAPS, 3-Tris (hydroxymethyl) methylamino-1-propanesulfonic acid.

The oligosaccharide chains in glycoconjugates such as glycoproteins and glycolipids have important roles in biological processes such as inflammatory and immunological responses, cell–cell recognition, cancer metastasis and fertilization in diverse eukaryotes (*1*, *2*). Oligosaccharide moieties are synthesized by a series of glycosyltransferases in cells (*3*, *4*). In humans, 110 genes encoding glycosyltransferases have been cloned so far (*5*). Recent progress has revealed that glycoproteins are present in prokaryotes as well, although the structures of bacterial *N*-linked sugars are different from the eukaryotic counterparts (*6*). Prokaryotes also have clusters of genes that encode glycosyltransferases and related enzymes for synthesis of various oligosaccharide chains (*7*). Several bacterial pathogens and symbionts have evolved specific oligosaccharide chains that mimic the host cell's surface carbohydrates, which are crucial for self/non-self recognition, to evade the immune system (*1*, *4*, *8*).

Studies of the relationship between structure and function of the oligosaccharide chains in glycoconjugates have contributed to the development of pharmaceutical products. For example, the addition of sialylated glycans to the erythropoietin, which has three sialylated glycans in the native form, extends the half-life of the erythropoietin in blood (*9*). Fully synthetic oligosaccharide vaccines have been tested for control of infections by bacteria such as *Haemophilus influenzae* type b (*10*), viruses such as human immunodeficiency viruses (*11*), and the protozoa that causes malaria (*11*). Carbohydrate microarrays have also been used to investigate the carbohydrate-binding specificities of bacteria, to detect pathogens during diagnosis, and to screen anti-adhesion therapeutics (*11*, *12*).

Terminal sugars play key roles in the function of carbohydrate chains. Sialic acid is usually found as an α-sialoside at non-reducing termini of carbohydrate chains in glycoconjugates. Thus, sialylated glycans are of particular interest in functional glycomics. In this context, an ample supply of various sialylated glycans and glycoconjugates is essential for basic research and the subsequent development of industrial products. Sialylated glycans can be synthesized either chemically or enzymatically, but enzymatic methods are generally preferred because the chemical synthesis

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of oligosaccharides requires complicated, multistep processes, and also because the stereospecific synthesis of α-sialosides is difficult in chemical methods.

Sialyltransferases transfer *N*-acetylneuraminic acid (NeuAc) from cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) to the non-reducing termini of the oligosaccharyl structures of various glycoconjugates. Although many sialyltransferases have been cloned from mammals (*13–19*), producing these enzymes in large quantity is difficult because mammalian glycosyltransferases are rarely expressed as active enzymes in *E. coli* (*14*). In general, bacterial enzymes are much more productive than mammalian ones in *E. coli* expression systems, and bacterial glycosyltransferases have a broad range of acceptor substrate specificities compared to mammalian glycosyltransferases. Thus, bacterial enzymes are indispensable tools in the preparation and modification of sialylated glycans (*20–24*).

To meet the demand for sialylated glycans, bacterial sialyltransferases with diverse substrate specificities, productivities, solubilities, stabilities and temperature ranges for activity are needed. Sialyltransferases are classified into five families in the Carbohydrate-Active enZymes (CAZy) database (http://www.cazy.org/) (*25*), and four of the families consist of bacterial enzymes: glycosyltransferase family 29, various sialyltransferases from eukaryotes and viruses; family 38, polysialyltransferases from bacteria such as *E. coli* (*26*) and *Neisseria meningitidis* (*27*); family 42, bacterial lipooligosaccharide sialyltransferases such as α2,3-sialyltransferase from *Campylobacter jejuni* (*28*) and α2,3/α2,8-sialyltransferase from *Haemophilus influenzae* (*29*); family 52, α2,3-sialyltransferases from bacteria such as *N. gonorrhoeae* and *N. meningitides* (*30*); *H. influenzae* (accession no. AAX88755), *Pasteurella multocida* (accession no. AAK02592); and family 80, α2,6-sialyltransferase from *Photobacterium damselae* (*31*), α2,3/α2,6-sialyltransferase from *P. multocida* (*23*), and *Haemophilus ducreyi* sequence (accession no. AAP95068). Further studies of new enzymes are necessary to expand the availability of tools for on-demand preparation of sialylated glycans.

We have been screening many marine bacteria for novel glycosyltransferase activity. Here, we report a novel α2,3-sialyltransferase, which uses both the α- and β-anomers of galactosides as acceptors, from a bacterium that belongs to the genus *Vibrio*. This is the first report of a sialyltransferase from the genus *Vibrio*.

MATERIALS AND METHODS

Screening of Bacteria—Bacteria were isolated on marine agar 2216 (Becton–Dickinson, Franklin Lakes, NJ) or nutrient agar (Becton–Dickinson) supplemented with 2% NaCl at 15, 25 or 30°C. Aliquots of the bacteria were suspended in 10% glycerol and stored at −80°C. Marine broth 2216 (6 ml; Becton–Dickinson) in a 15-ml test tube was inoculated with a bacterial isolate and cultivated at 15, 25 or 30°C for 18 h on a rotary shaker (180 rpm). Bacteria were harvested from 2 to 4 ml of the culture broth by centrifugation, suspended in 200 μl of 20 mM sodium cacodylate buffer (pH 6.0) containing 0.2% Triton X-100, lysed by sonication on ice, and measured immediately for sialyltransferase activity.

Sialyltransferase Assay—The reaction mixture (30 μl) consisted of a sample of enzyme, 120 mM lactose, 2.3 mM CMP-NeuAc (Nakarai Tesque, Kyoto, Japan), 4620 Bq CMP-[4,5,6,7,8,9-^{14}C]-NeuAc (Amersham Biosciences, Little Chalfont, UK), 100 mM bis–Tris buffer (pH 6.0), 0.5 M NaCl and 0.03% Triton X-100. The reaction and measurement were performed according to the procedure described by Yamamoto *et al.* (*32*). One unit (U) was defined as an activity transferring 1 μmol NeuAc to the lactose in 1 min.

Sialylation of Pyridylaminated Lactose—The reaction mixture (20 μl) consisting of a sample of sialyltransferase, 1.25 μM CMP-NeuAc, 2.5 μM pyridylaminated lactose (PA-Sugar Chain 026; Takara Biochemicals, Shiga, Japan), 15 mM bis–Tris buffer (pH 6.0) and 0.1% Triton X-100 was incubated at 25°C for 14 h. The mixture was then applied to a PALPAK Type R analytical column (0.46 cm × 25 cm; Takara Biochemicals) that was equilibrated with 100 mM acetic acid–triethylamine buffer (pH 5.0) containing 0.15% *n*-butanol in an HPLC (LC10A; Shimadzu Corp., Kyoto, Japan). The column temperature was kept at 40°C, and the flow rate was 1 ml/min. The concentration of *n*-butanol was increased linearly by 0.01%/min from 0.15 to 0.5%. Pyridylaminated carbohydrates were detected by fluorescence (Ex: 320 nm; Em: 400 nm). The retention time of pyridylaminated lactose, pyridylaminated 6'-sialyllactose, which was prepared using α2,6-sialyltransferase from *P. damselae* (*32*), and pyridylaminated 3′-sialyllactose (PA-Sugar Chain 029; Takara Biochemicals) were 4.00, 4.39 and 5.40 min, respectively.

Sialidase Activity—The 15 μl reaction mixture was composed of a sample of enzyme, 1.7 μM pyridylaminated 3′-sialyllactose, 15 mM bis–Tris buffer (pH 6.0) and 0.1% Triton X-100 and was incubated at 25°C for 0.5, 3, 15 and 48 h. The mixture was analysed with the PALPAK Type R analytical column under the conditions described earlier.

Southern Hybridization—Unless stated otherwise, the methods used for molecular cloning and DNA manipulation were those described by Sambrook *et al.* (*33*). Genomic DNA was isolated from a bacterial pellet using Genomic-tip 100/G (Qiagen Inc., Chatsworth, CA). DNA was digested with restriction endonuclease *Eco*RI or *Hin*dIII (Takara Biochemicals), separated by 0.7% agarose gel electrophoresis, and blotted on a Hybond N$^+$ nylon membrane filter (Amersham Biosciences) by alkaline-blotting with 0.4 M NaOH. A 929-bp *Eco*RI fragment from the *Photobacterium phosphoreum* JT-ISH-467 α2,3-sialyltransferase gene (Tsukamoto, H., Takakura, Y. and Yamamoto, T., in preparation) was labelled with [α-^{32}P]-dCTP using a Multiprime DNA labelling system (Amersham Biosciences), and used as a probe. Hybridization was performed in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA at 55°C for 18 h. The membrane was washed twice in 40 mM sodium phosphate (pH 7.2), 5% SDS and 1 mM EDTA at 55°C for 15 min.

Genomic Library and Screening—The genomic DNA was partially digested with *Sau*3AI

(Takara Biochemicals). The DNA fraction between 9 and 16 kb was prepared by ultracentrifugation and ligated into the *Bam*HI site of λDASHII (Stratagene, La Jolla, CA). The resultant DNA was packaged with Gigapack III Gold extract (Stratagene) to yield the JT-FAJ-16 genomic library. Plaques from the genomic library were transferred to Hybond-N^+ and hybridized to the 929-bp probe from the JT-ISH-467 α2,3-sialyltransferase gene. Hybridization was performed using an ECL direct labelling and detection system (Amersham Biosciences), according to the manufacturer's instructions with minor modifications. Hybridization was performed at 37°C and the wash at 50°C.

Cloning of a DNA Fragment Containing the α2,3-Sialyltransferase Gene—Phage DNA was isolated with a Lambda Mini Kit (Qiagen Inc.), digested with *Eco*RI and separated on an agarose gel. Southern analysis was performed as described earlier. The 3.6 kb *Eco*RI fragment was subcloned into pBluescript SK(–) (Stratagene).

DNA Sequencing—DNA sequences were determined by the dideoxy chain termination method (*34*) with an ABI PRISM fluorometric autocycle sequencer (model 310 Genetic Analyzer; Applied Biosystems, Foster City, CA). The sequence was determined on both strands. DNA and amino acid sequences were analysed by Genetyx ver. 7.0 (Genetyx Co., Tokyo, Japan), and a database search was performed using the BLAST program in DDBJ and GenBank.

Construction of Expression Cassettes—For amplification of the α2,3-sialyltransferase gene, three primers, VST3F1, VST3F2 and VST3R1 (Table 1), were designed so as to create *Bsp*HI (VST3F1, VST3F2) and *Bam*HI (VST3R1) restriction sites at the ends of PCR products. For the full-length form, VST3F1 and VST3R1 were used for PCR, and for the N-terminal truncated form, VST3F2 and VST3R1 were used. The PCR reaction mixture (50 μl) consisted of 300 ng of the template DNA (the 3.6-kb *Eco*RI fragment), 50 pmol of primers, 4 μl of each 2.5 mM dNTP, 2.5 units of *ExTaq* DNA polymerase and 5 μl of 10 × *ExTaq* buffer, according to the manufacturer's instructions (Takara Biochemicals). The reaction was hot-started at 96°C for 3 min; incubated at 96°C for 1 min, 50°C for 1 min and 72°C for 2 min for 10 cycles; and further incubated at 72°C for 6 min in a Program Temp Control System (Astek, Fukuoka, Japan). The PCR product was cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The PCR products were digested with *Bsp*HI (New England Biolabs, Ipswich, MA) and *Bam*HI (Takara Biochemicals), and cloned between the *Nco*I (Takara Biochemicals) and *Bam*HI sites of an expression vector pTrc99A to give plasmid pV16-ST3-FL and pV16-ST3-△N. The expression cassettes were introduced into the *E. coli* strain TB1.

Table 1. **Primers used in this study.**

Primer	Sequence[a] (5′-3′)
VST3F1	TGGATAACTC*ATGA*AAAACATT ATAACAAAAAGAATG
VST3F2	TATTATCGTC*ATGA*ACAATGAT AACAGCACTACC
VST3R1	TCTTTTTAGGATCC*TTA*AAATGTC GCTGATTAGTTTTAT
27f	AGAGTTTGATCCTGGCTCAG
1525r	AAAGGAGGTGATCCAGCC

[a]Primers were designed to create restriction sites (underlined) or start and stop codons (italicized).

Expression of Recombinant Protein in E. coli—A single colony of *E. coli* harbouring an expression cassette was inoculated with LB broth (Becton–Dickinson) containing 100 μg/ml ampicillin at 30°C until the A_{600} value reached 0.5. Then, 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Wako Pure Chemicals, Osaka, Japan) was added to the culture, which was further incubated at 30°C for 4 h. The bacterial pellet collected by centrifugation was dissolved in 20 mM bis–Tris buffer (pH 7.0) containing 0.5 M NaCl and 0.3% Triton X-100 and was sonicated on ice. The supernatant was used as the crude enzyme solution in further analyses.

16S rRNA Gene—For amplification of 16S ribosomal RNA gene, primers 27f and 1525r (Table 1) were designed on the basis of eubacterial 16S rRNA genes (*35*). Polymerase chain reaction (PCR) was done with genomic DNA as a template as described earlier, except for annealing at 55°C, elongation for 1 min and a cycle number of 30. The PCR product was cloned into the pCR2.1 TOPO vector for sequencing.

Purification of Recombinant α2,3-Sialyltransferase—Escherichia coli—TB1 harbouring pV16-ST3-△N was shaken in 3 ml of LB broth containing 100 μg/ml ampicillin at 30°C for 16 h. Then, 300 ml of the same medium containing 1 mM IPTG was inoculated with the seed culture and incubated for 16 h. Bacteria were harvested by centrifugation from 10.8 l culture to yield a pellet. The pellet was suspended in 16 ml of 20 mM bis–Tris buffer (pH 6.0) containing 0.3% Triton X-100 (Buffer A) per gram of pellet and lysed by sonication on ice. Cellular debris was removed by centrifugation at 100,000 × *g* for 60 min, and the supernatant was filtered through a 0.45 μm cellulose acetate membrane. The filtrate was fractionated on a HiLoad 26/10 Q Sepharose HP column (2.6 cm × 10 cm; Amersham Biosciences) equilibrated with Buffer A in a protein liquid chromatography system (ÄKTA; Amersham Biosciences). The enzyme was eluted with a linear gradient from 0 to 1 M NaCl in Buffer A. The fractions with sialyltransferase activity were pooled, diluted with 20 mM potassium phosphate buffer (pH 6.0) containing 0.3% Triton X-100 (Buffer B), and applied to a hydroxyapatite column (Bio-Scale CHT20-I; 1.5 cm × 11.3 cm; Bio-Rad, Hercules, CA) equilibrated with Buffer B. The enzyme was eluted with a linear gradient of potassium phosphate from 20 to 500 mM. The active fractions were pooled, diluted with Buffer A and loaded on a Mono Q 10/100 GL column (1 cm × 10 cm; Amersham Biosciences) equilibrated with Buffer A. The enzyme was eluted with a linear gradient of NaCl from 0 to 1 M in Buffer A, and the active fractions were collected. All of the chromatographic purification steps were performed at 7°C.

Protein Concentration Determination and SDS–PAGE—Protein concentration was determined with Coomassie Protein Assay Reagent (Pierce Chemical, Rockford, IL) and a modified Lowry method with bovine serum albumin as a calibration standard.

SDS–PAGE (*36*) was performed using precast polyacrylamide gels (Atto, Tokyo, Japan) and molecular mass standard samples (Bio-Rad), and the gels were stained with Coomassie Brilliant Blue R-250.

Mass Spectrometry—Matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on 10–20 pmol of purified protein with an AXIMA-CFR MALDI-TOF mass spectrometer (Shimadzu Corp.) operated in the linear mode with delayed extraction of ions, using 10 mg/ml sinapinic acid as the matrix in 0.1% trifluoroacetic acid saturated with 50% acetonitrile.

pH and Temperature Profile—The reaction in the enzyme assay was performed at 25°C for 3 min as described earlier, except that 100 mM bis–Tris buffer (pH 6.0) was replaced with 100 mM sodium acetate buffer (pH 4.0–5.0), 100 mM sodium cacodylate buffer (pH 5.0–7.0), 100 mM phosphate buffer (pH 7.0–8.0), 100 mM 3-Tris (hydroxymethyl) methylamino-1-propanesulfonic acid buffer (TAPS; pH 8.0–9.0). For the temperature profile, sialyltransferase activity was measured at 5, 10, 15, 20, 25, 30, 35, 40 and 45°C for 3 min.

Acceptor Substrate Specificity—The following monosaccharide and oligosaccharide acceptor substrates were examined: methyl-α-D-galactopyranoside (Sigma, St Louis, MO), methyl-β-D-galactopyranoside (Sigma), methyl-α-D-glucopyranoside (Sigma), methyl-β-D-glucopyranoside (Sigma), methyl-α-D-mannopyranoside (Fluka, Germany), *N*-acetylgalactosamine (Sigma), *N*-acetyglucosamine (Sigma), lactose (Wako Pure Chemicals), Gal-β-1,3-GlcNAc-β-OMe (Larodan, Malmo, Sweden) and *N*-acetyllactosamine (Seikagaku Kogyo, Tokyo, Japan). The reaction in the sialyltransferase assay protocol was modified so that the 24 μl reaction mixture was composed of a sample of purified sialyltransferase, 42 mM acceptor substrate, 911 μM CMP-NeuAc, 403 Bq CMP-[4,5,6,7,8,9-^{14}C]-NeuAc, 20 mM sodium cacodylate buffer (pH 5.5), 0.5 M NaCl and 0.04% Triton X-100. The following pyridylaminated (PA)-sugar chains were purchased from Takara Biochemicals: globotriose, asialo GM1-trisaccharide, asialo GM1-tetrasaccharide, Forssman pentasaccharide, GM1-pentasaccharide, GD1b-hexasaccharide, *N*-acetyllactosamine type, *N*-acetyllactosamine type monosialylated biantennary, lacto-*N*-neotetraose, lacto-*N*-tetraose, lacto-*N*-fucopentaose II (Lewis X) and lacto-*N*-fucopentaose III. The reaction in the enzyme assay for PA-sugars was performed as described earlier, except that lactose was replaced with various PA-sugar chains.

Reaction with Asialo-fetuin and Asialo-mucin—Asialofetuin or asialo-bovine submaxillary mucin was used as an acceptor substrates in the sialyltransferase assay. After the reaction, protein or mucin was purified by gel filtration using a Sephadex G-50 superfine column (0.8×18 cm^2; Amersham Biosciences), and subjected to scintillation counting.

Electrospray Ionization Mass Spectrometry (ESIMS), and ^{1}H- and ^{13}C-Nuclear Magnetic Resonance Spectroscopy—The reaction mixture (300 μl) composed of 100 mM benzyl-β-D-galactopyranoside, 67 mM CMP-NeuAc, 4.7 mU/μl purified recombinant truncated sialyltransferase, 0.5 M NaCl and 20 mM bis–Tris buffer (pH 6.0) was incubated at 30°C for 15 min and then loaded on a Sephadex G-15 column (2.8×74 cm; Amersham Biosciences). The product was eluted with H_2O (400 μl/min) and analysed by ESIMS. The fractions that contained the reaction product were pooled, concentrated *in vacuo*, resuspended in H_2O and loaded on a Cosmosil 75C18-OPN column (2.6×12 cm; Nakarai Tesque). The product was eluted with H_2O (400 μl/min). After the column chromatography, each fraction was analysed by ESIMS spectroscopy. The fractions that contained the reaction product were pooled, concentrated *in vacuo*, resuspended in D_2O and analysed by ^{1}H- and ^{13}C-NMR spectroscopy.

RESULTS

Isolation of a Marine Bacterium with α2,3-Sialyltransferase Activity—More than 3000 isolates of marine bacteria were examined for sialyltransferase activity, and 10 were found to have that activity. We focused on one bacterium, JT-FAJ-16, isolated from the guts of a horse mackerel fish, because we thought that this bacterium might be a *Vibrio* sp., which have so far not been reported to have sialyltransferase activity. JT-FAJ-16 was tested for the ability to transfer sialic acid to pyridylaminated lactose. Crude extracts from this bacterium yielded pyridylaminated 3'-sialyllactose, indicating that it had α2,3-sialyltransferase activity. Its morphology and physiological properties suggested that this bacterium was a member of the Vibrionaceae family. Furthermore, a partial DNA sequence of the 16S ribosomal RNA gene (accession no. AB308043) was clearly classified into the *Vibrio* genus cluster and showed 99.5% identity to that of *Vibrio rumoiensis* (accession no. AB013297). These results suggest that this bacterium belongs to the genus *Vibrio* and is closely related to *V. rumoiensis* (*37*). Therefore, we designated this bacterium as *Vibrio* sp. JT-FAJ-16.

Confirming the Existence of an α2,3-Sialyltransferase Gene Homologue—We previously cloned the α2,3-sialyltransferase gene from a Vibrionaceae bacterium, *Photobacterium phosphoreum* JT-ISH-467 (Tsukamoto, H., Takakura, Y. and Yamamoto, T., in preparation). To investigate whether there is a homolog of this gene in the JT-FAJ-16 genome, Southern blot analysis was performed. Both a 3.6-kb *Eco*RI and a 7-kb *Hin*dIII DNA fragment of JT-FAJ-16 hybridized to the JT-ISH-467 α2,3-sialyltransferase gene, indicating that there is a homologue of the α2,3-sialyltransferase gene in the JT-FAJ-16 genome.

Cloning of the α2,3-Sialyltransferase Gene Homologue—The genomic library of JT-FAJ-16 was constructed and screened with the JT-ISH-467 α2,3-sialyltransferase gene probe. Twelve plaques were selected and purified. The λ DNAs from each plaque were isolated, digested with *Eco*RI and subjected to Southern blot analysis. The 3.6-kb *Eco*RI fragment that hybridized to the probe was cloned into pBluescript and the complete DNA sequence was determined. An open reading frame (ORF) of 1209 bp (accession no. AB308042) was found in the cloned DNA, and it encoded a protein of 402 amino acids (Fig. 1). The N-terminal hydrophobic region

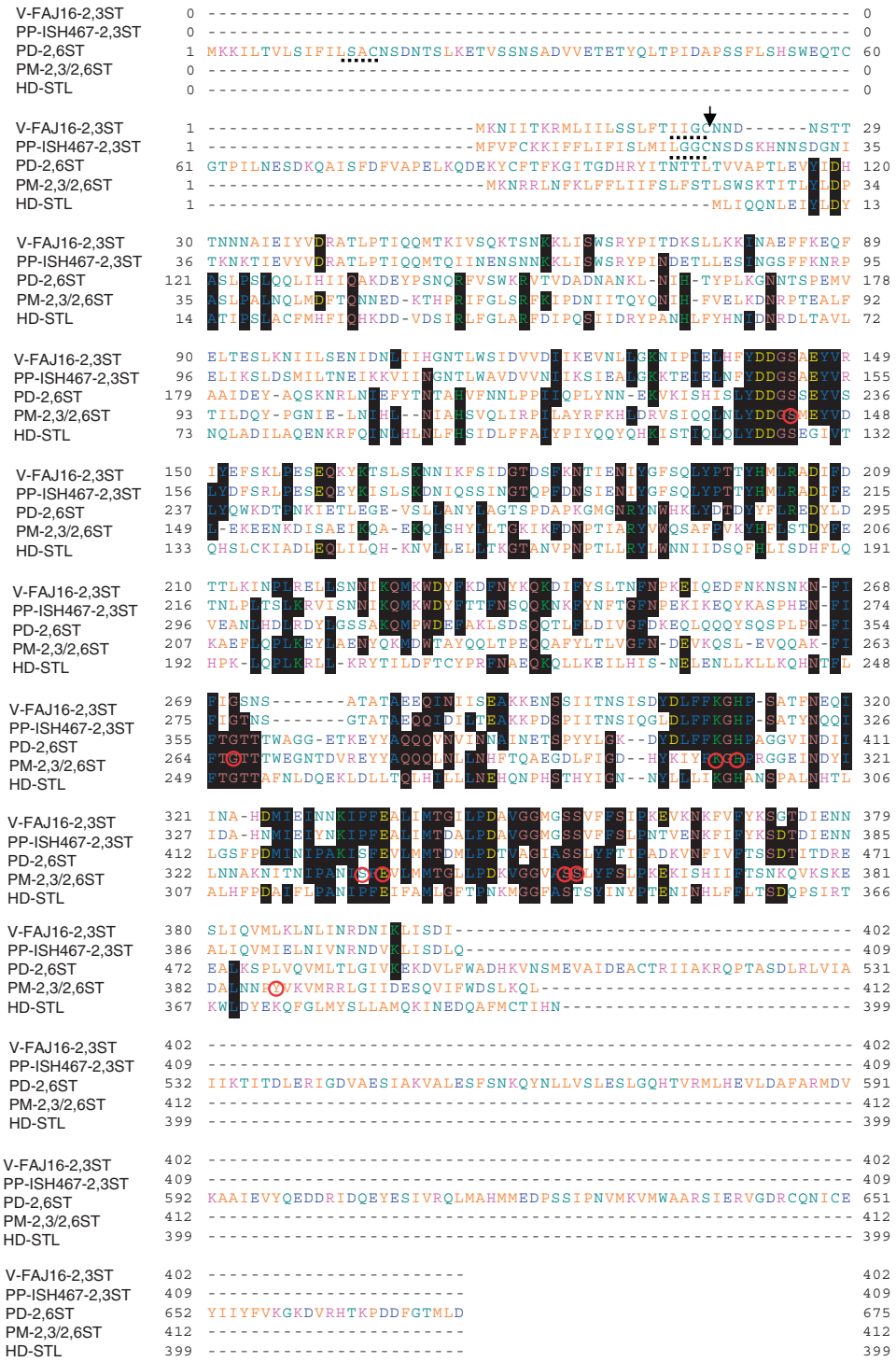

Fig. 1. **Multiple alignments among the bacterial sialyltransferases in the GT family 80.** The amino acid sequences from *Vibrio* sp. JT-FAJ-16 α/β-galactoside α2,3-sialyltransferase (V-FAJ16-2,3ST, accession no. AB308042), *P. phosphoreum* JT-ISH-467 α/β-galactoside α2,3-sialyltransferase (PP-ISH467-2,3ST, accession no. BAF63530), *P. damselae* β-galactoside α2,6-sialyltransferase (PD-2,6ST, accession no. BAA25316), *Pasteurella multocida* multifunctional sialyltransferase (PM-2,3/2,6ST, accession no. AAY89061) and *Haemophilus ducreyi* hypothetical protein (HD-STL, accession no. AAP95068) were aligned using Genetyx ver. 7.0. The conserved amino acids are boxed in black. The arrow shows the cleavage site for the truncated form of *Vibrio* sp. JT-FAJ-16 α2,3-sialyltransferase. Red circles indicate amino acids relating to the CMP-binding site (*38*). The dot underlines show lipobox sequences (*39*).

consisting of 22 amino acids was predicted to be a signal peptide by Genetyx ver. 7.0. The molecular mass and pI of the N-terminal truncated form of the protein (Asn^{23}-Ile^{402}) were calculated as 43,734 and 5.48, respectively.

The DNA sequence of the ORF had 69.7 and 51.2% identity to the sequences of α2,3-sialyltransferase from *P. phosphoreum* JT-ISH-467 (accession no. AB293982) and α2,6-sialyltransferase from *P. damselae* (accession no. AB012285), respectively. The deduced amino acid sequence had 64.7, 30.5 and 27.3% identity to the sequences of α2,3-sialyltransferase from *P. phosphoreum* JT-ISH-467 (accession no. BAF63530), α2,6-sialyltransferase from *P. damselae* (accession no. BAA25316) and α2,3/α2,6-sialyltransferase from *Pasteurella multocida* (accession no. AAY89061), respectively. It showed no significant homology to other sialyltransferases in the analysis by Genetyx ver. 7.0. Thus, this protein belongs to GT family 80 in the CAZy database. The multiple alignments among the bacterial sialyltransferases in the GT family 80 is shown in Fig. 1. Seven of the nine residues that make up the CMP-binding site in a multifunctional sialyltransferase from *P. multocida* (*38*) were conserved in the JT-FAJ-16 sequence. JT-FAJ-16 had a lipo box motif (*39*) in the N-terminal region (Ile^{19} to Cys^{22}).

Expression of α2,3-Sialyltransferase in E. coli—Expression cassettes of the full-length form in pV16-ST3-FL and the truncated form (N-terminal Lys^{2} to Cys^{22} deletion) in pV16-ST3-△N were constructed and expressed in *E. coli*. The crude enzyme extracts from both *E. coli 6*strains treated with IPTG showed sialyltransferase activity. Sialyltransferase activity of pV16-ST3-△N was much higher than that of pV16-ST3-FL, and reached 215.7 U/l *E. coli* culture. The crude enzyme extract from the truncated form was reacted with pyridylaminated lactose, and the products were analysed by HPLC. The retention time of the products was consistent with an authentic pyridylaminated 3′-sialyllactose, indicating that the cloned gene encodes a protein with α2,3-sialyltransferase activity.

Purification of Recombinant α2,3-Sialyltransferase—The truncated recombinant enzyme produced in *E. coli* harbouring pV16-ST3-△N was purified 103-fold by three chromatography steps in the presence of detergent, with a yield of 5.4% (Table 2). The enzyme migrated as a single band on SDS–PAGE (Fig. 2). The specific activity of the purified enzyme was 57.5 U/mg. The molecular mass of the truncated enzyme was 43,921 kDa by MALDI-TOF MS.

Mass Spectrometry and NMR Spectroscopy of the Reaction Products—The truncated recombinant enzyme was reacted with benzyl-β-D-galactopyranoside as the acceptor substrate, and the reaction product was purified by the combination of ion-exchange chromatography and gel-filtration chromatography. The purified reaction product was analysed by ESIMS and ^{1}H- and ^{13}C-NMR spectroscopy. The [M-H]-ion peak of the enzymatic reaction product was at 559.9 in the mass spectrum, indicating that the product was sialylbenzyl-β-D-galactopyranoside, which has an exact mass of 561.21. The structural reporter group signals by ^{1}H-NMR of the enzymatic reaction product were as follows: δ 4.56 ppm (Gal β H-1; 4.52 ppm*), δ 3.58 ppm (Gal β H-2; 3.55 ppm*), δ 4.08 ppm (Gal β H-3, 4.10 ppm*), δ 3.96 ppm (Gal β H-4; 3.94 ppm*), δ 2.76 ppm (NeuAc H-3 eq; 2.75 ppm*), δ 1.79 ppm (NeuAc H-3 ax; 1.79 ppm*), δ 3.68 ppm (NeuAc H-4; 3.65 ppm*), δ 2.03 ppm (N-Ac; 2.02 ppm*). The structural reporter group signals by ^{13}C-NMR of the enzymatic reaction product were as follows: δ 69.36 ppm (Gal β C-2; 69.86 ppm*), δ 76.72 ppm (Gal β C-3, 76.03 ppm*), δ 67.80 ppm (Gal β C-4; 68.00 ppm*), δ 39.93 ppm (NeuAc C-3; 40.19 ppm*), δ 68.71 ppm (NeuAc C-4; 68.79 ppm*). Asterisks mean the values

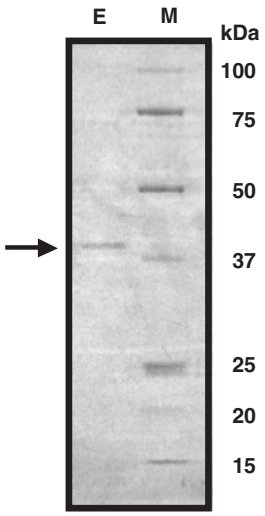

Fig. 2. **SDS–PAGE analysis of the purified recombinant α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16.** The truncated enzyme was purified, and subjected to SDS–PAGE using a 12.5% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. The arrow shows the purified enzyme. E, a lane for enzyme; M, a lane for molecular weight marker.

Table 2. **Purification scheme for recombinant α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16.**

Step	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Cell extract[b]	4170	2330	0.56	100	1
Q Sepharose	251	404	1.61	17	3
Hydroxyapatite	10.5	305	29.1	13	52
MonoQ	2.2	127	57.5	5.4	103

[a]One unit (U) was defined as an activity transferring 1μmol NeuAc to the lactose in 1 min.
[b]From 10.8-l culture.

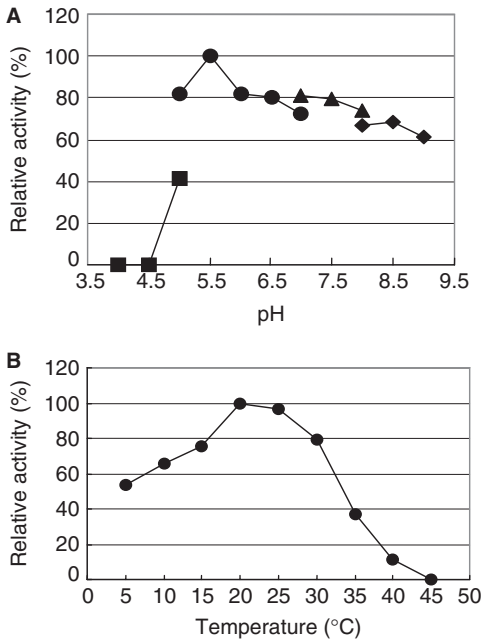

Fig. 3. **The effect of pH (A) and temperature (B) on the activity of recombinant α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16**. The enzymatic activity is shown relative to the maximum value. A. The enzyme assay was performed as described in 'Materials and Methods' section at 25°C in 100 mM sodium acetate buffer (squares), 100 mM sodium cacodylate buffer (circles), 100 mM phosphate buffer (triangles) or 100 mM TAPS buffer (diamonds). The activity at pH 5.5 is 100% value. (B) The enzyme assay was performed as described in 'Materials and Methods' section in 20 mM bis–Tris buffer (pH 6.0) at various temperatures. The activity at 20°C is 100% value.

from an α2,3-sialoside reported by Sabesan and Paulson (*40*). Thus, the chemical shift data by ^{1}H- and ^{13}C-NMR showed good agreement with that reported for an α2,3-sialoside. These results further confirmed that the enzyme was an α2,3-sialyltransferase.

Properties of α2,3-Sialyltransferase—The truncated recombinant enzyme was active (more than 60% of full activity) at pH 5.0–9.0 (Fig. 3A) and at 10 to 30°C (Fig. 3B). It was optimally active at pH 5.5 and at 20°C (Fig. 3). The enzyme was twice as active in the presence of 500–700 mM NaCl, and it showed no sialidase activity. The enzyme had a broad acceptor substrate range; it was able to transfer NeuAc to all of the sugars examined (Tables 3 and 4). Interestingly, the enzyme used both the α-amoner and the β-anomer of the sugars. For monosaccharides, methyl-α-D-galactopyranoside was the most preferred acceptor substrate, followed by methyl-β-D-galactopyranoside (Table 3). The relative activities of methyl-α/β-D-galactopyranosides were twice those of methyl-α/β-D-glucopyranosides, methyl–D-mannopyranoside, *N*-acetylgalactosamine, or *N*-acetylglucosamine (Table 3). For disaccharides, the enzyme transferred NeuAc to *N*-acetyllactosamine, methyl-β-D-galactopyranosyl-β-1,3-*N*-acetylglucosamine and lactose with high efficacy (Table 3). When pyridylaminated lactose, pyridylaminated GM1-pentasaccharide or pyridylaminated GD1b-hexasaccharide was used as an acceptor substrate, a single peak with the same retention time as that of an authentic GM3-Neu5Ac-trisaccharide (NeuAcα 2-3Galβ1-4Glc-PA), GD1a-hexasaccharide [(NeuAcα2-3)Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-PA] or GT1b-heptasaccharide [(NeuAcα2-3)Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glc-PA] was produced, respectively (Table 4). The enzyme also transferred NeuAc to both asialo-fetuin (*N*-linked type) and asialo-mucin (*O*-linked type).

DISCUSSION

We found a novel sialyltransferase from the *Vibrio* sp. bacterium, JT-FAJ-16. Bacterial sialyltransferases have been reported from the genera *Neisseria* (*30*, *41*, *42*), *Campylobacter* (*28*), *Haemophilus* (*29*, *43*, *44*), *Pasteurella* (*23*), *Escherichia* (*26*, *45*), and *Photobacterium* (*31*, *32*). A sialyltransferase has not been ever purified from the genus *Vibrio*, although the NeuAcα2-3Gal and NeuAcα2-6Gal linkages were found in the capsular polysaccharides of *Vibrio cholerae* strain H11 (*46*). This is the first report of a sialyltransferase from the genus *Vibrio*.

Sialyltransferases usually transfer NeuAc to the galactose at the non-reducing terminus of sugar chains. The acceptor substrate specificity of mammalian sialyltransferases is generally high, and enzymes are specific for the type of the sugars and linkages. In contrast, bacterial sialyltransferases are less specific, which may allow various sialylated glycans to be prepared. The α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16 uses various sugars as acceptors (Tables 3 and 4). One of the most striking features is that this enzyme acts on both methyl-α-D-galactopyranoside and methyl-β-D-galactopyranoside. Similar specificity has been reported in the α2,3-sialyltransferase from *Neisseria meningitides* immunotype L1 (*42*). Since the α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16 transfers NeuAc to both the α-anomer and the β-anomer of sugars, this enzyme can be described as an α/β-galactoside α2,3-sialyltransferase.

The most preferred acceptor substrate of the enzyme was methyl-α-D-galactopyranoside among the tested monosaccharides and disaccharides (Table 3). It seems that there is no significant difference among the activities with lactose, *N*-acetyllactosamine and methyl-β-D-galactopyranosyl-β-1,3-*N*-acetylglucosaminide (Table 3), which suggests that this α/β-galactoside α2,3-sialyltransferase is not sensitive to the nature of the second sugar from the non-reducing terminus and to the linkage between the terminal two sugars. In contrast, β-galactoside α2,6-sialyltransferase from *P. damselae* (*32*) is active mainly on the β-anomer, and shows selectivity for both the second sugar from the non-reducing terminus and the linkage. The relative activities of methyl-α/β-D-galactopyranosides were twice those of methyl-α/β-D-glucopyranosides (Table 3), suggesting that affinity of the enzyme for the acceptor substrate is affected by the orientation of the hydroxyl group at the C-4 position of the sugar. Also, the relative activity of methyl-α-D-galactopyranoside was twice that of *N*-acetylgalactosamine and methyl-β-D-mannopyranoside,

Table 3. **The transfer assay of recombinant α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16 for monosaccharide and disaccharides.**

Acceptor substrate		Sialyltransferase activity	
Name	Structure	NeuAc transferred (nmol/min)	Relative activity[a] (%)
Methyl-α-D-galactopyranoside	Galα*O*Me	10.3	135
Methyl-β-D-galactopyranoside	Galβ*O*Me	8.7	114
Methyl-α-D-glucopyranoside	Glcα*O*Me	4.7	62
Methyl-β-D-glucopyranoside	Glcβ*O*Me	4.0	52
Methyl-β-D-mannopyranoside	Manα*O*Me	4.3	57
N-Acetylgalactosamine	GalNAc	4.4	58
N-Acetylglucosamine	GlcNAc	4.2	55
Lactose	Galβ1-4Glc	7.6	100
N-Acetyllactosamine	Galβ1-4GlcNAc	9.6	127
Methyl–D-galactopyranosyl-β-1, 3-*N*-Acetylglucosamine	Galβ1-3GlcNAcβ*O*Me	9.4	124

[a]Lactose is 100% value.
The assay was conducted as described in 'Materials and Methods'.

Table 4. **The transfer assay of recombinant α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16 for various pyridylaminated carbohydrate chains.**

Acceptor substrate	Structure	No. of products
Lactose	*Galβ1-4Glc-PA	1(GM3-NeuAc-trisaccharide)
Globotriose	Galα1-4Galβ1-4Glc-PA	1
Asialo GM1-trisaccharide	GalNAcβ1-4Galβ1-4Glc-PA	1
Asialo GM1-tetrasaccharide	Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA	2
Forssman pentasaccharide	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-PA	1
GM1-pentasaccharide	*Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-PA	1(GD1a-hexasaccharide)
GD1b-hexasaccharide	*Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glc-PA	1(GT1b-heptasaccharide)
N-Acetyllactosamine type, biantennary	(Galβ1-4αGlcNAcβ1-2Manα1-6) (Galβ1-4αGlcNAcβ1-2Manα1-3) Manβ1-4αGlcNAcβ1-4GlcNAc-PA	3
N-Acetyllactosamine type, monosialylated biantennary	(NeuAcα2-6Galβ1-4αGlcNAcβ1-2Manα1-6)(Galβ1-4αGlcNAcβ1-2 Manα1-3)Manβ1-4αGlcNAcβ1-4GlcNAc-PA	1
Lacto-*N*-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	1
Lacto-*N*-tetraose	Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	1
Lacto-*N*-fucopentaose II	Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc-PA	1
Lacto-*N*-fucopentaose III	Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-PA	1

Asterisk shows the sialylation sites predicted by HPLC. Products in parentheses were predicted by comparison of the HPLC retention time with that of an authentic sample, and the structure is shown in the text.
The reaction mixture was described in 'Materials and Methods'. The mixture was then applied to an analytical column. Pyridylaminated carbohydrates were detected by fluorescence.

suggesting that the enzyme might be affected by the acetoamido group at the C-2 position and by the orientation of the hydroxyl group at the C-2 position. The enzyme was also allowed to react with various oligosaccharides, and some possible reaction products were predicted on the basis of HPLC retention times (e.g. GD1a-hexasaccharide in Table 4). Future work will include the purification and identification of these products.

The primary structure of the α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16 shows 27–65% identity to the primary structure of the sialyltransferases in GT family 80 in the CAZy database. To date, GT family 80 has been reported only in species of Vibrionaceae [*Photobacterium* (*31*; Tsukamoto, H., Takakura, Y. and Yamamoto, T., in preparation) and *Vibrio* (this work)] and Pasteurellaceae (*Pasteurella* (*23*) and *Haemophilus*) in γ-proteobacteria. On the other hand, other GT families have been found widely in β-, γ-, ε-proteobacteria and Gram-positive bacteria (http://www.cazy.org/). Thus, GT family 80 enzymes may be originated from a common ancestral gene that evolved after the γ-proteobacteria had genetically separated from other groups of proteobacteria.

The amino acids that relate to the binding site of CMP (*38*), a key component of the donor substrate for sialic acid, were appeared to be well conserved among the sialyltransferases (Fig. 1), although Tyr-388 in the *P. multocida* sialyltransferase, which participates in the formation of hydrogen bonds to the terminal phosphate oxygen of CMP through a water molecule was not conserved. The α/β-galactoside α2,3-sialyltransferase

from *Vibrio* sp. JT-FAJ-16 had a lipo box motif (*39*) (Ile^{19} to Cys^{22}) in the N-terminal region (Fig. 1). The presence of a cysteine in the lipo box at the N-terminus of the enzyme is characteristic of lipoproteins that localize to the periplasmic space and associate with lipids in the bacterial transmembrane (*39*, *47*). Sialyltransferases from *P. damselae* and *P. phosphoreum* have this motif, but a multifunctional sialyltransferase from *P. multocida* does not (Fig. 1). The function of this lipobox motif in the JT-FAJ-16 native enzyme remains to be elucidated.

The productivity of the truncated recombinant enzyme reached 215.7 U/l *E. coli* culture. This value is comparable to that (224.5 U/l *E. coli* culture) of recombinant α2,6-sialyltransferase from *P. damsela* (*31*). The specific activity was 57.5 U/mg protein, when lactose was used as an acceptor substrate. This value is one of the highest among bacterial sialyltransferases, which range from 0.05 U/mg for *N. gonorrhoeae* (*22*) to 1.44 U/mg for *N. meningitides* (*42*) to 5.5–7.0 U/mg for *P. damsela* (*31*, *32*) to 60 U/mg for *P. multocida* (*23*). Unlike an α2,3/α2,6-sialyltransferase from *P. multocida*, the *Vibrio* sp. JT-FAJ-16 enzyme had no sialidase activity, indicating the usefulness of this enzyme.

In this article, we confirmed that the α/β-galactoside α2,3-sialyltransferase from *Vibrio* sp. JT-FAJ-16 has very broad acceptor specificity, high productivity, high specific activity and no sialidase activity. These properties make this sialyltransferase a powerful tool for the study of glycotechnology, especially in producing various α/β-sialosides and modifying glycoconjugates.

We thank Dr Yasuhiro Kajihara for identifying the reaction product by ESIMS and NMR spectroscopy. We also thank Ms Hitomi Kajiwara and Ms Masako Ichikawa for their excellent technical assistance.

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers: AB308042 and AB308043.

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