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# *Photobacterium* sp. JT-ISH-224 Produces Two Sialyltransferases, α-/β-Galactoside α2,3-Sialyltransferase and β-Galactoside α2,6-Sialyltransferase

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**A novel bacterium, *Photobacterium* sp. JT-ISH-224, that produces α-/β-galactoside α2,3-sialyltransferase and β-galactoside α2,6-sialyltransferase, was isolated from the gut of a Japanese barracuda. The genes that encode the enzymes were cloned from the genomic library of the bacterium using the genes encoding α-/β-galactoside α2,3-sialyltransferase from *P. phosphoreum* and β-galactoside α2,6-sialyltransferase from *P. damselae* as probes. The nucleotide sequences were determined, and open reading frames of 1,230 and 1,545 bp for encoding an α2,3-sialyltransferase and an α2,6-sialyltransferase of 409- and 514-amino acid residues, respectively, were identified. The α2,3-sialyltransferase had 92% amino acid sequence identity with the *P. phosphoreum* α2,3-sialyltransferase, whereas the α2,6-sialyltransferase had 54% amino acid sequence identity with the *P. damselae* α2,6-sialyltransferase. For both enzymes, the DNA fragments that encoded the full-length protein and its truncated form lacking the putative signal peptide sequence were amplified by a polymerase chain reaction and cloned into an expression vector. Each gene was expressed in *Escherichia coli*, and the lysate from each strain had enzymatic activity. The α2,3-sialyltransferase catalysed the transfer of *N*-acetylneuraminic acid (NeuAc) from CMP-NeuAc to lactose, α-methyl-galactopyranoside and β-methyl-galactopyranoside with low apparent $K_m$ and the α2,6-sialyltransferase catalysed the transfer of NeuAc from CMP-NeuAc to lactose with low apparent $K_m$.**

**Key words: bacterial sialyltransferase, glycosyltransferase family 80, photobacterium, sialic acid.**

Abbreviations: bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2(hydroxymethyl)-1,3-propanediol; Bn, benzyl; bp, base pairs; CMP-NeuAc, cytidine monophosphate *N*-acetylneuraminic acid; Fuc, fucose; Gal, galactose; Glc, glucose; HPLC, high performance liquid chromatography; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Me, methyl; [M-H]−, deprotonated molecular ion; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; NAc, *N*-acetyl; NaCl, sodium chloride; NeuAc, *N*-acetylneuraminic acid; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electorophoresis; PCR, polymerase chain reaction; ph, phenyl.

Terminal sugars play key roles in the functions of glycoconjugates such as glycoproteins and glycolipids. Sialic acid is often found as an α-sialoside at non-reducing termini of carbohydrate chains in glycoconjugates. Thus, sialylated glycans may contribute to recognition and are of particular interest in the functional glycomics of processes such as inflammatory and immunological responses, cell–cell recognition and cancer metastasis in diverse eukaryotes (*1*, *2*). In prokaryotes, the terminal sialic acid is important during interactions with hosts. It is also involved in protecting viruses such as influenza A and B (*1*) and bacteria such as *Haemophilus influenzae* (*3*) from the host immune system.

At present, the range of available sialylated glycans is very limited. If sialylated glycans can be easily synthesized and prepared, functional research will progress rapidly. Enzymatic sialylation of galactosides is generally superior, whereas the chemical addition of sialic acid to oligosaccharides requires complicated multistep processes. Further, the stereospecific synthesis of α-sialosides is difficult. Sialyltransferases that have been cloned from mammals are not easy to produce in large quantities because mammalian glycosyltransferases are rarely expressed as active enzyme forms in recombinants. On the other hand, bacterial enzymes are much more productive in recombinant expression systems, and bacterial glycosyltransferases have much broader acceptor–substrate specificities than do mammalian glycosyltransferases. Thus, bacterial enzymes are expected to serve as indispensable tools in the preparation and modification of sialylated glycans (*4*–*7*).

Bacterial sialyltransferases are needed in a variety of forms with respect to substrate specificity, pH and temperature range for activity, productivity, solubility and other enzymatic properties. Five families of

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sialyltransferases are represented in the CAZy (carbohydrate-active enzymes) data base (http://www.cazy.org/). These families differ in their structurally related catalytic and carbohydrate-binding modules (*8*). In eukaryotes and viruses, one family, glycosyltransferase family 29 (*9–12*), consists of various sialyltransferases that have a sialyl-motif domain in their amino acid sequences (*13*). The other four of the families consist of bacterial enzymes: family 38, polysialyltransferases from bacteria such as *Escherichia coli* (*14*) and *Neisseria meningitidis* (*15*); family 42, lipooligosaccharide α2,3-sialyltransferases, α2,3/α2,8-sialyltransferases, from bacteria such as *Campylobacter jejuni* (*16*) and *H. influenzae*; family 52, α2,3-sialyltransferases, α2,3/α2,6-sialyltransferases from bacteria such as *H. influenzae* (*17*), *H. ducreyi* (*18*), *N. meningitidis* (*19*) and *Pasteurella multocida* and family 80, α2,6-sialyltransferases, α2,3-sialyltransferases, α2,3/α2,6-sialyltransferases from bacteria such as *P. multocida* (*6*), *H. ducreyi*, *Photobacterium damselae* (*20*), *P. phosphoreum* (*21*), *P. leiognathi* (*22*) and *Vibrio* sp. (*23*). However, the extent of the diversity is still far from the desired level, and the search for new enzymes must continue until the sialylated glycans of interest can be prepared on demand.

In this study, we surveyed bacteria for novel glycosyltransferase activities and hereby report the cloning of the genes for α-/β-galactoside α2,3-sialyltransferase and β-galactoside α2,6-sialyltransferase, from a marine bacterium, *Photobacterium* sp. JT-ISH-224 that was isolated from the gut in Japanese barracuda (*Sphyraena pinguis*), and characterization of the recombinant enzymes produced from the cloned genes.

## MATERIALS AND METHODS

*Screening of Bacteria*—Samples of seawater, soil, small animals and seaweed were collected from various coastal locations in Japan. Bacteria that grew on marine agar 2216 (BD Bioscience) or nutrient agar (BD Bioscience) that was supplemented with 2% NaCl at 15, 25 or 30°C were isolated from the samples. Six millilitres of marine broth 2216 (BD Bioscience) 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) that contained 0.2% Triton X-100, lysed by sonication on ice and measured immediately for sialyltransferase activity. The reaction was carried out at 25°C for 2 h. The cultured bacteria were suspended in 10% glycerol and stored at −80°C.

*Sialyltransferase Assay*—The 30 μl reaction mixture consisted of lysate, crude or purified enzyme, 120 mM lactose, 2.3 mM CMP-NeuAc (Nacalai Tesque), 4,620 Bq CMP-[4,5,6,7,8,9-$^{14}$C]-NeuAc (Amersham Biosciences), 20 mM bis-Tris buffer (pH 6.0), 0.5 M NaCl and 0.03% Triton X-100. The reaction was carried out at 30°C. After the reaction, the reaction mixture was diluted to 2 ml with 5 mM potassium phosphate buffer (pH 6.8), then applied to a column (0.5 × 2 cm) of AG1-X2 resin (Bio-Rad) equilibrated in phosphate form with 1 M potassium phosphate buffer (pH 6.8) (*20*). The eluate (2 ml) was collected and added to the scintillation mixture Ultima Gold from Packard. The mixture was measured directly in a liquid scintillation counter (model TR 1900; Packard). One unit (U) of activity was defined as the amount of enzyme that transferred 1 μmol of NeuAc per min to lactose at 30°C and pH 5.0.

*Sialylation of Pyridylaminated Lactose*—The 20 μl reaction mixture was composed of lysate, crude or purified sialyltransferase, 1.25 μM CMP-NeuAc, 2.5 μM pyridylaminated lactose (PA-Sugar Chains 026, Takara Bio), 15 mM bis-Tris buffer (pH 6.0) and 0.1% Triton X-100 and incubated at 25°C for 24 h. The measurements were performed according to the procedure (*21*), using a PALPAK type R analytical column (0.46 × 25 cm; Takara Bio). The retention times for pyridylaminated lactose, pyridylaminated 6′-sialyllactose [prepared using α2,6-sialyltransferase from *P. damselae* JT0160 (*20*)], and pyridylaminated 3′-sialyllactose (PA-Sugar Chains 029, Takara Bio) were 4.00, 4.39 and 5.40 min, respectively.

*Southern Analysis of Genomic DNA*—Unless mentioned otherwise, the methods used for molecular cloning and DNA manipulation were those described by Maniatis *et al*. (*24*). Genomic DNA was isolated from bacteria using Genomic-tip 100/G (Qiagen). The genomic DNA digested with *Eco*RI or *Hin*dIII was electorophoresed on 0.7% agarose gel, then transferred to Hybond-N+ (Amersham Biosiences) and hybridized to a probe DNA labelled with $^{32}$P using a random prime labelling system (Rediprime II, Amersham Biosciences) in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA at 55°C for 18 h. The probe was either the 929-bp DNA fragment of the α2,3-sialyltransferase gene from *P. phosphoreum* JT-ISH-467 (*21*) or the 1.2 kb *Eco*RI–*Hin*dIII DNA fragment of the α2,6-sialyltransferase gene from *P. damselae* JT0160 (*25*). 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.

*Isolation of Homologues from Genomic Library*—The genomic DNA was partially digested with *Sau*3AI. The fragments between 9 and 16 kb and the *Bam*HI arms of λ DASHII (Stratagene) were ligated and packaged with Gigapack III Gold packaging extract (Stratagene). Plaques from the genomic library were screened by plaque hybridization, which was performed by the same protocol as the genomic Southern hybridization. Clones were characterized by restriction analysis and Southern hybridization. DNA fragments were subcloned in pBluescript SK(−) (Stratagene).

*Construction of Expression Cassettes*—For amplification of DNA fragments, primers (Table 1) were designed to create restriction sites at the ends of the PCR products. The fragment was synthesized by 15 cycles of PCR using a pair of primers in the same conditions described previously (*21*). The PCR product was digested with restriction enzymes and cloned with expression vector pTrc99A (Amersham Biosiences).

*16S rRNA Gene*—The 16S rRNA gene was amplified using primers 27f and 1525r (Table 1) (*26*) from the genomic DNA by PCR in the same conditions as described previously (*21*).

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

| Primer | Sequence |
|---|---|
| PS3STF1[a] | 5′-AAG GGA ATA CAT GTT CGT TTT TTG TAA AAA AAT G-3′ |
| PS3STF2[a] | 5′-GGG ATG TAC CAT GGA CTC TAA TCA CAA TAA CTC AG-3′ |
| PS3STR1[a] | 5′-ATT AAA ATG GAT CCT TAC TGC AAA TCA CTT ATC AAC-3′ |
| PS6STF1[a] | 5′-AGA ATA TCA TGA AAA ACT TTT TAT TAT TAA C-3′ |
| PS6STF2[a] | 5′-CTT GTA ACA TGT CAG AAG AAA ATA CAC AAT C-3′ |
| PS6STR1[a] | 5′-TTT TTT GGA TCC CTA GAC TGC AAT ACA AAC ACC-3′ |
| 27f | 5′-AGA GTT TGA TCC TGG CTC AG-3′ |
| 1525r | 5′-AAA GGA GGT GAT CCA GCC-3′ |

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

*Phylogenetic Analysis*—Sequences were aligned by the Clustal W program (version 1.83) (*27*) and then drawn using the program Genetyx (version 7.0.10). Gaps were omitted manually from the phylogenetic analysis. Neighbour-joining analysis was also performed using Clustal W (*28*) with the algorithm of Kimura's two-parameter method (*29*). Bootstrap confidence values were obtained with 10,000 resamplings (*30*).

*Recombinant Sialyltransferases*—For preparing the recombinant sialyltransferase, *E. coli* TB1 that harboured the constructed plasmid was shaken in 3 ml Luria-Bertani (LB) broth (Type Miller, BD Bioscience) containing 100 μg/ml ampicillin at 30°C for 16 h. Then, 300 ml of the same medium that contained 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was inoculated with the seed culture and incubated for 16 h. Bacteria were harvested by centrifugation 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 per gram of pellet, then 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 enzyme was prepared according to the procedure used to purify the protein from *P. phosphoreum* (*21*).

*Amino Acid Sequence and Mass Spectrometry*—The $NH_2$ terminus of the purified protein was sequenced with a Procise 494 cLC Protein Sequencing System (Applied Biosystems). Matrix-assisted laser desorption mass spectrometry was performed with 10–20 pmol of purified protein with a AXIMA-CFR MALDI-TOF MS (Shimadzu) 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 Profiles*—For the pH profile, the reaction in the purified recombinant truncated enzyme assay protocol was carried out at 30°C as described earlier except that 20 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 TAPS [3-Tris (hydroxymethyl)methylamino-1-propanesulfonic acid] buffer (pH 8.0–9.0), 100 mM CHES (2-*N*-cyclohexylamino ethanesulfonic acid) buffer (pH 9.0–10.0) or 100 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer (pH 10.0–11.0). For the temperature profile, the sialyltransferase assay protocol was carried out at 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50°C for 3 min. The reactions were within the range of linearity, and not >15% of either CMP-NeuAc or lactose was consumed in any assay. Assays were performed in triplicate.

*Acceptor Specificity*—Acceptor substrates examined were monosaccharides and oligosaccharides as follows: methyl-α-D-galactopyranoside (Sigma-Aldrich), methyl-β-D-galactopyranoside (Sigma-Aldrich), *N*-acetyl-D-galactosamine (Sigma-Aldrich), lactose (Wako Pure Chemicals), Gal-β-1,3-GlcNAc-β-OMe (Larodan), *N*-acetyllactosamine (Seikagaku Kogyo), Gal-α-1,3-Gal-α-OMe (Glycorex), Gal-β-1,3-Gal-β-OMe (Glycorex) and Fuc-α-1,2-Gal-β-1,4-Glc (Dextra Laboratories). The reaction in the sialyltransferase assay protocol was modified so that 24 μl of the mixture was composed of 50 μU/μl purified sialyltransferase, 8.3 mM or 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 reaction was then run at 30°C for 0.5, 1 or 2 min in triplicate.

*Kinetic Studies for Acceptor Substrates*—The acceptor substrates lactose, methyl-α-D-galactopyranoside and methyl-β-D-galactopyranoside were tested in the reaction in the sialyltransferase assay protocol. The mixture (30 μl) was composed of 4.2 mM CMP-NeuAc, 4,290 Bq CMP-[4,5,6,7,8,9-$^{14}$C]-NeuAc, various concentrations of an acceptor substrate, 5.9 μU/μl of the purified sialyltransferase, 115 mM sodium cacodylate buffer (pH 5.0), 520 mM NaCl and 0.02% Triton X-100. The reaction was carried out in triplicate at 30°C for 3 min. The kinetic parameters, $K_m$ and $V_{max}$, were obtained from a linear least squares regression analysis of a Hanes–Woolf plot.

*Sialidase Activity*—The 15 μl of reaction mixture was composed of 1 mU of purified 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 by the PALPAK type R analytical column under the same conditions described earlier.

*Protein Determination*—Coomassie Protein Assay Reagent (Pierce) and a modified Lowry method were used according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

*SDS-PAGE*—Precast polyacrylamide gels (Atto) and molecular mass standard samples (Bio-Rad) were used and stained with Coomassie brilliant blue R-250.

*Electrospray Ionization Mass Spectroscopy (ESI-MS), 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, 5 mU/μl of 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 loaded on a Sephadex G-15 column (2.8 × 74 cm; Sigma-Aldrich). The product was eluted with $H_2O$ (400 μl/min) and analysed by ESI-MASS spectroscopy. The fractions that contained the reaction product were pooled, concentrated

*in vacuo*, resuspended in $H_2O$ and loaded on a Cosmosil $75C_{18}$-OPN column (2.6 × 12 cm; Nacalai Tesque). The product was eluted with $H_2O$ (400 μl/min). After the ODS column chromatography, each fraction was analysed by ESI-MS. The fractions that contained the reaction product were pooled, concentrated *in vacuo*, resuspended in $D_2O$ and analysed with $^1H$ and $^{13}C$ NMR spectroscopy as in a previous report (*31*).

## RESULTS

*Isolation and Identification of Marine Bacteria Producing Sialyltransferase*—More than 3,000 isolates of bacteria were examined for sialyltransferase activity, and 10 of these tested positive in the enzymatic assay. We focused on strain, JT-ISH-224, isolated from the gut of the Japanese barracuda (*S. pinguis*), in this study. In a test for activity in the transfer of NeuAc to pyridylaminated lactose, JT-ISH-224 had α2,3-sialyltransferase and α2,6-sialyltransferase activities that yielded two products, pyridylaminated 3′-sialyllactose and 6′-sialyllactose. The 16S rRNA gene of JT-ISH-224 was partially sequenced (1,500 bp, accession number AB293986) and was highly homologous (99.2 and 99.1% identity) with the 16S rRNA gene from *P. phosphoreum* ATCC11040 (the type strain) (accession number X74687) and from *P. iliopiscarium* ATCC51760 (the type strain) (accession number X74687), respectively. Optimum bacterial growth was observed on nutrient agar supplemented with 1–3% NaCl and to some extent at 5% but not at 0.5%. The bacterium grew optically on marine agar 2216 plate at 25–30°C and grew to some extent at 4°C and 32.5°C but not at 35°C. Because these characteristics did not correspond to the two type strains (*32*), we concluded that strain JT-ISH-224 is closely related to but is neither *P. phosphoreum* nor *P. iliopiscarium*. We are further investigating the identity of the bacterium.

*Cloning, Expression and Purification of α2,3-Sialyltransferase*—Genomic Southern analysis showed that a 16 kb *Eco*RI fragment and 2.7 kb and 5 kb *Hin*dIII fragments hybridized with the DNA fragment for the α2,3-sialyltransferase gene from *P. phosphoreum* JT-ISH-467 as a probe, showing that JT-ISH-224 had a homologue of the α2,3-sialyltransferase of *P. phosphoreum* JT-ISH-467. Approximately, several thousand clones from the λDASHII library of strain JT-ISH-224 were screened with the probe, and six hybridization-positive clones were identified. After plaque purification and rescreening, DNA was prepared from each clone and characterized by restriction analysis and by Southern hybridization. Three clones were chosen for further characterization. DNA fragments of 2.7 and 5.0 kb were identified in a *Hin*dIII digest and cloned into the pBluescript SK(−). An open reading frame of 1,230 bp (accession number AB293984) that was found in the clone encoded a protein of 409 amino acids (Fig. 1) with a calculated molecular mass of 46,594 Da. The $Leu^{19}$–$Cys^{22}$ sequence was identical to the lipobox sequence of *E. coli* (*33*).

For amplification of the homologue of the α2,3-sialyltransferase gene, three primers (PS3STF1, PS3STF2 and PS3STR1) (Table 1) had *Pci*I, *Nco*I and *Bam*HI restriction sites, respectively, at the ends of the PCR products. The full-length of the homologue was synthesized using PS3STF1 and PS3STR1. The PCR product was digested with *Pci*I and *Bam*HI, and cloned between the *Nco*I and *Bam*HI sites of expression vector pTrc99A to yield the plasmid pPS-ST3-FL. A truncated homologue was amplified using PS3STF2 and PS3STR1, digested with *Nco*I and *Bam*HI and cloned between the *Nco*I and *Bam*HI sites of pTrc99A to yield pPS-ST3-Δ2–24. Expression cassettes of the homologue of sialyltransferase in pPS-ST3-FL and of the truncated form, which lacks the $NH_2$ terminal hydrophobic region, in pPS-ST3-Δ2–24 were constructed and expressed in *E. coli*. In Triton X-100, the lysates from *E. coli* that harboured plasmid pPS-ST3-FL and pPS-ST3-Δ2–24 were examined for sialyltransferase activity, and both were positive in the enzymatic assay. They were tested for activity to transfer NeuAc to pyridylaminated lactose and had α2,3-sialyltransferase activity yielding pyridylaminated 3′-sialyllactose but not pyridylaminated 6′-sialyllactose. It was concluded that the cloned homologue coded α2,3-sialyltransferase.

Although only 2 and 5% of the activity in the lysate from *E. coli* with plasmid pPS-ST3-FL and pPS-ST3-Δ2–24, respectively, were retained in the supernatants without detergent compared to the activity before centrifugation, 60 and 95% of the activity from that with plasmid pPS-ST3-FL and pPS-ST3-Δ2–24, respectively, were present with 0.3% Triton X-100. The truncated genes produced proteins with greater enzymatic activity than did the full-length genes. The truncated enzyme was purified 384-fold through the four chromatographic steps in the presence of the detergent with a yield of 5.6% (Table 2). The enzyme migrated as a single polypeptide with a molecular mass of ~40 kDa in electrophoresis under denaturing conditions. The molecular mass of the enzyme was 43,995 Da (accuracy 1,000 p.p.m.) in MALDI-TOF MS, very close to the mass of the predicted enzyme (44,027 Da) composed of 386 amino acid residues. The sequence of the $NH_2$ terminal amino acid of the recombinant enzymes from *E. coli* that harboured plasmid pPS-ST3-Δ2–24 was determined to be MDSNHNNSDGNITKNK. The $NH_2$ terminal amino acid sequence was identical to the sequence from the 1st to 16th amino acid of the primary translation product of the gene.

*Cloning, Expression and Purification of α2,6-Sialyltransferase*—Genomic Southern analysis showed that a 12.5 kb *Eco*RI fragment and a 9 kb *Hin*dIII fragment hybridized with the fragment of the α2,6-sialyltransferase gene from *P. damselae* JT0160 as a probe, demonstrating that JT-ISH-224 has a homologue of the α2,6-sialyltransferase. Eight hybridization-positive clones were identified from the λDASHII library of strain JT-ISH-224. By Southern hybridization, three clones were chosen for further characterization. DNA fragments of 3.5 kb were identified in an *Nhe*I digest and cloned into the pBluescript SK(−) digested with *Xba*I. An open reading frame of 1,545 bp (accession number AB293985) in the clone was found to encode a protein of 514 amino acids (Fig. 1) with a calculated molecular

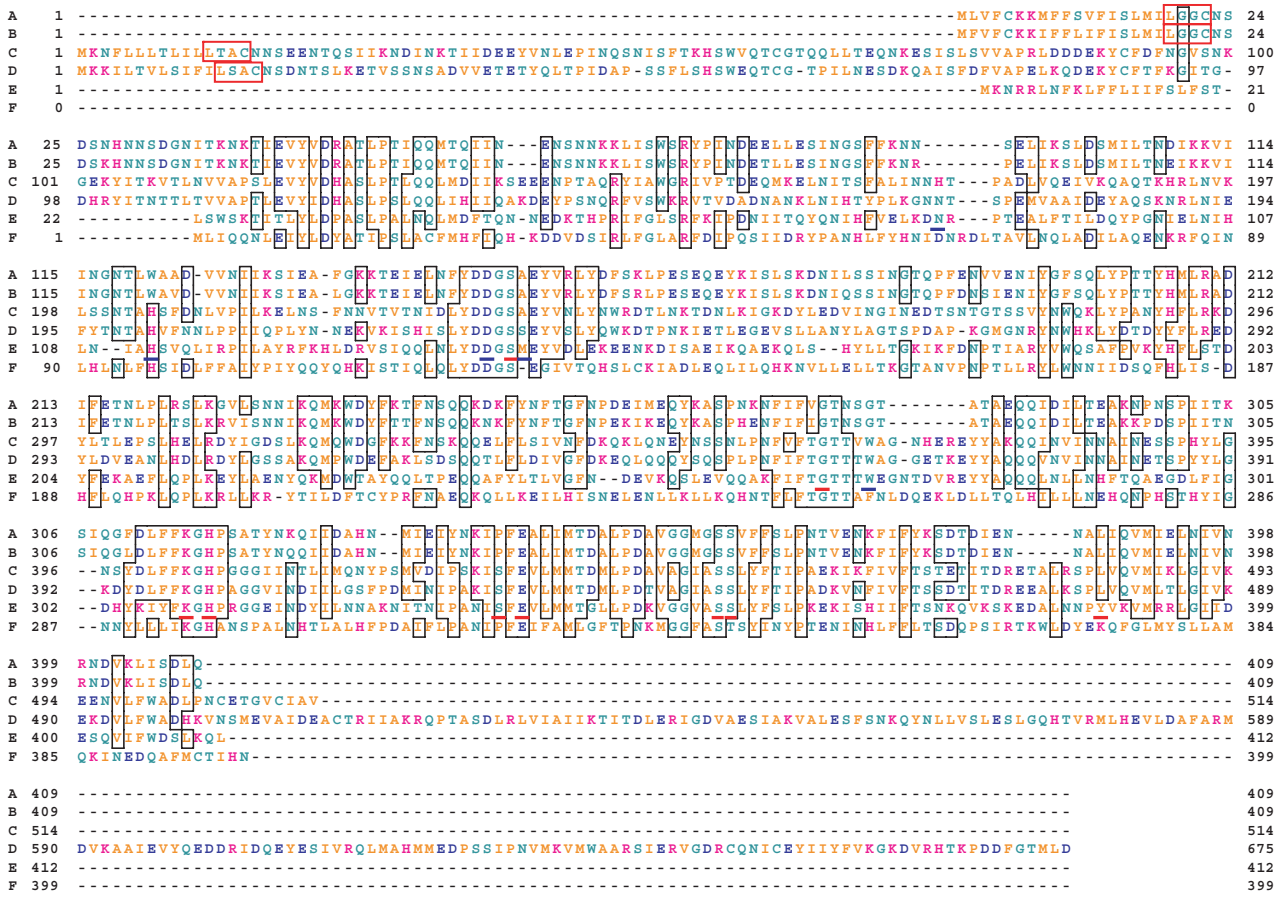

Fig. 1. **Multiple alignment of sialyltransferases from *Photobacterium* sp. JT-ISH-224 and GT family 80.** (A) α-/β-galactoside α2,3-sialyltransferase (accession number BAF92025) and (C) β-galactoside α2,6-sialyltransferase (BAF92026) from *Photobacterium* sp., (B) α-/β-galactoside α2,3-sialyltransferase (BAF63530) from *P. phosphoreum*, (D) α2,6-sialyltransferase (BAA25316) from *P. damselae*, (E) multifunctional sialyltransferase (AAY89061) from *Pasteurella multocida* and (F) the hypothetical protein HD0053 (AAP95068) from *Haemophilus ducreyi* were aligned using the Clustal W alignment program (version 1.83, DDBJ). The conserved amino acid positions in boxes were determined and the program Genetyx (version 7.0.10). The sequence in the red box is identical to the lipobox sequence of Gram-negative bacteria. Red underlined amino acids are CMP-binding site. Blue underlined amino acids are lactose-binding sites.

Table 2. **Purification of the recombinant, truncated α-/β-galactoside α2,3-sialyltransferase expressed in *Escherichia coli*.**

| Chromatography step | Volume (ml) | Total protein (mg) | Total activity (U[a]) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Crude extract[b] | 840 | 4025 | 234 | 0.058 | 100 | 1.0 |
| Q sepharose | 70 | 242 | 40.4 | 0.17 | 17 | 2.9 |
| Hydroxyapatite | 32 | 24 | 76.8 | 3.21 | 33 | 55 |
| Mono Q | 3 | 1.6 | 26.2 | 16.6 | 11 | 285 |
| Superdex | 7.5 | 0.59 | 13.2 | 22.4 | 5.6 | 384 |

[a]One unit (U) of activity was defined as the amount of enzyme that transferred 1 μmol of *N*-acetylneuraminic acid (NeuAc) per min to lactose at pH 5 and 30°C in the standard assay described in MATERIALS AND METHODS section. [b]Crude extract was prepared from 61 g wet cells grown in 8.11 culture.

mass of 58,518 Da. The $Leu^{12}$–$Cys^{15}$ sequence was identical to the lipobox sequence.

For amplification of the homologue of α2,6-sialyltransferase gene of *P. damselae* JT0160, three primers (PS6STF1, PS6STF2 and PS6STR1) (Table 1) had *Bsp*HI, *Nco*I and *Bam*HI restriction sites, respectively, at the ends of the PCR products. With primers PS6STF1 and PS6STR1, the full-length of the homologue of α2,6-sialyltransferase gene was cloned in plasmid pPS-ST6-FL, and with primers PS6STF2 and PS6STR1, the truncated homologue was cloned in plasmid pPS-ST6-Δ2–17. Expression cassettes of the full-length homologue of sialyltransferase in pPS-ST6-FL and of the truncated form, which lacks the $NH_2$ terminal hydrophobic region, in pPS-ST3-Δ2–17 were constructed and expressed in *E. coli*. They were then tested for

Table 3. **Purification of the recombinant, truncated β-galactoside α2,6-sialyltransferase expressed in *Escherichia coli*.**

| Chromatography step | Volume (ml) | Total protein (mg) | Total activity (U[a]) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Crude extract[b] | 238 | 1100 | 13167 | 12.0 | 100 | 1.0 |
| Q sepharose | 64 | 371 | 11794 | 31.8 | 90 | 2.7 |
| Hydroxyapatite | 180 | 138 | 9448 | 68.3 | 72 | 5.7 |
| Mono Q | 4.5 | 24 | 2720 | 113 | 21 | 9.4 |

[a]One unit (U) of activity was defined as the amount of enzyme that transferred 1 μmol of NeuAc per min to lactose at pH 5 and 30°C in the standard assay described in MATERIALS AND METHODS section. [b]Crude extract was prepared from 15 g wet of cells grown in 2.7 l culture.

activity in the transfer of NeuAc to pyridylaminated lactose and had α2,6-sialyltransferase activity, yielding pyridylaminated 6′-sialyllactose but not pyridylaminated 3′-sialyllactose. It was concluded that the cloning homologue coded α2,6-sialyltransferase.

Although only 5 and 8% of the activity with plasmid pPS-ST6-FL and pPS-ST6-Δ2–17, respectively, were retained in the supernatant without detergent compared to the activity before centrifugation, 60 and 96% of the activity from that with plasmid pPS-ST6-FL and pPS-ST6-Δ2–17, respectively, were present with 0.3% Triton X-100. The truncated genes produced greater enzymatic activity than did the full-length genes. The truncated enzyme was purified 9.4-fold through the three chromatographic steps in the presence of the detergent with a yield of 21% (Table 3). The enzyme migrated as a single polypeptide with a molecular mass of ~55 kDa during electrophoresis under denaturing conditions. The molecular mass of the enzyme was 56,523 Da (accuracy 1,000 p.p.m.) in MALDI-TOF MS. The value was very close to 56,732 Da for the predicted enzyme with 498 amino acid residues. The sequence of the $NH_2$ terminal amino acid of the recombinant enzymes from *E. coli* with plasmid pPS-ST6-Δ2–17 was determined to be SEENTQSIIKNDIN. The $NH_2$ terminal amino acid sequence was identical to the sequence from the 2nd to 15th amino acid of the primary translation product of the gene. The first amino acid, Met, may have been processed.

*Phylogenetic Analysis*—The amino acid sequence of the α2,3-sialyltransferase from JT-ISH-224 had 92, 34, 33, 29 and 19% identity to the following respective GT family 80 enzymes: α-/β-galactoside α2,3-sialyltransferase (protein ID BAF63530) in *P. phosphoreum* JT-ISH-467 (*21*), α2,6-sialyltransferase (BAF92026) in *Photobacterium* sp. JT-ISH-224, β-galactoside α2,6-sialyltransferase (BAA25316) in *P. damselae* JT0160 (*25*), β-galactoside α2,3/α2,6-sialyltransferase (AAK02272) in *Pasteurella multocida* subsp. *multocida* Pm70 (*6*) and the hypothetical protein (AAP95068) in *H. ducreyi* 35000HP. The residues that had been identified as sites for binding to CMP in the sialyltransferase from *P. multocida* (*34*) appeared to be conserved in the α2,3-sialyltransferase from JT-ISH-224 (Fig. 1). On the other hand, the protein had no homology with sialyltransferases in the other families. Thus, this enzyme can be classified in GT family 80 in the CAZy data base.

A homology search showed that the amino acid sequence of the α2,6-sialyltransferase from JT-ISH-224 had 54, 36, 35 and 23% identity to GT family 80 enzymes: β-galactoside α2,6-sialyltransferase (BAA25316) in *P. damselae* JT0160, β-galactoside α2,3/α2,6-sialyltransferase (AAK02272) in *Pasteurella multocida* subsp. *multocida* Pm70, α-/β-galactoside α2,3-sialyltransferase (BAF63530) in *P. phosphoreum* JT-ISH-467 and the hypothetical protein (AAP95068) in *H. ducreyi* 35000HP, respectively. Because the CMP-binding sites were conserved in the α2,6-sialyltransferase from JT-ISH-224, this enzyme can also be classified in GT family 80 (Fig. 1).

As described in Fig. 2, phylogenic analysis revealed that the six sialyltransferases in GT family 80 were clustered into three groups with high bootstrap confidence values. The two α2,3-sialyltransferases from *Photobacterium* sp. fell into the same cluster. Likewise, the two α2,6-sialyltransferases from *Photobacterium* sp. fell into one group, and one sialyltransferase from *Pasteurella multocida* and one homologue *H. ducreyi* fell into the other. The three clusters were a trichotomy.

*General Properties of α2,3- and α2,6-Sialyltransferases*—Temperatures between 25°C and 30°C and pH values between 5.0 and 5.5 were optimal for the purified truncated recombinant α2,3- and α2,6-sialyltransferases. The α2,3-sialyltransferase was about 1.5 times as active in the presence of NaCl at 500 and 750 mM as in the absence of NaCl, whereas the 2,6-sialyltransferase was about 1.1 times as active in the presence of NaCl at 500 and 750 mM as in the absence of NaCl.

The purified α2,3- and α2,6-sialyltransferases were examined for sialylation activity for pyridylaminated lactose, and the production of pyridylaminated 3′-sialyllactose and 6′-sialyllactose, respectively, was confirmed. However, prolonged incubation of the reaction of α2,3-sialyltransferase resulted in a slight gradual decrease of the product, indicating that the enzyme also had a very weak sialidase activity. Then, the enzyme was assayed for degradation of pyridylaminated 3′-sialyllactose, and a low level of sialidase activity was indeed present. After 0.5, 3, 15 and 48 h incubation, 0, 0.03, 0.6 and 1.9% of pyridylaminated 3′-sialyllactose, respectively, were converted to pyridylaminated lactose with the α2,3-sialyltransferase from *Photobacterium* sp. JT-ISH-224, whereas 0.7, 4, 16 and 24% of the pyridylaminated 3′-sialyllactose, respectively, were converted to pyridylaminated lactose with the α2,3-sialyltransferase from *P. phosphoreum* JT-ISH-467 prepared as described in a previous paper (*21*). No sialidase activity was detected for the α2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224.

The purified truncated enzyme of the α2,3-sialyltransferase was able to transfer NeuAc to all of the examined

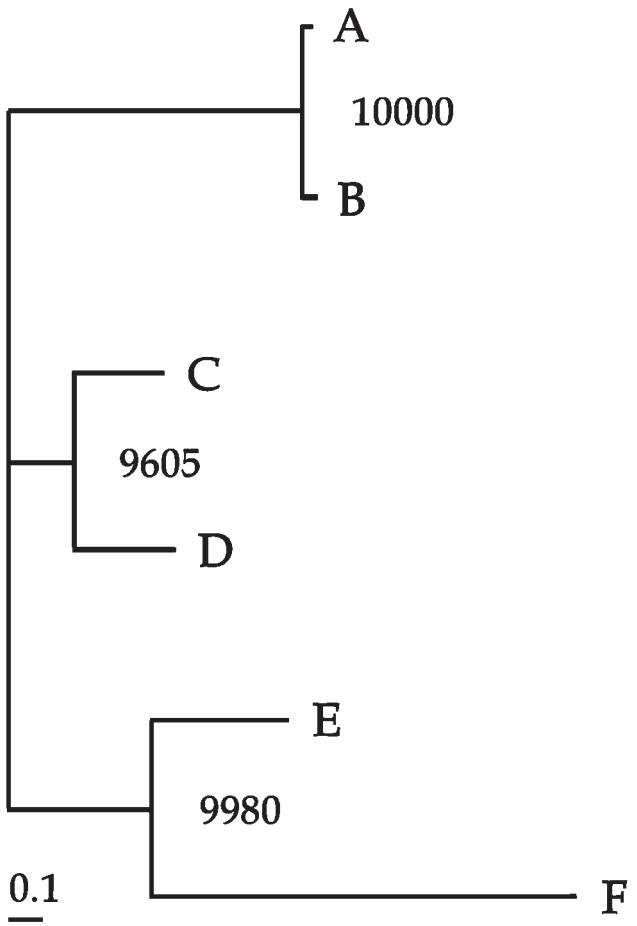


Fig. 2. **Phylogeny of sialyltransferases from *Photobacterium* sp. JT-ISH-224 and GT family 80.** The neighbour-joining analysis was performed using the Clustal W alignment program (version 1.83, DDBJ). Phylogenetic tree was deduced from the amino acid sequences of (A) α/β-galactoside α2,3-sialyltransferase (accession number BAF92025) and (C) β-galactoside α2,6-sialyltransferase (BAF92026) from *Photobacterium* sp., (B) α/β-galactoside α-2,3-sialyltransferase (BAF63530) from *P. phosphoreum*, (D) α2,6-sialyltransferase (BAA25316) from *P. damselae*, (E) multifunctional sialyltransferase (AAY89061) from *Pasteurella multocida* and (F) the hypothetical protein HD0053 (AAP95068) from *Haemophilus ducreyi*. Scale bar represents 0.1 substitutions per amino acid position. Bootstrap confidence values obtained with 10,000 resamplings are given at the branch points.

saccharides (Tables 4 and 5) and pyridylaminated lactose. Methyl-α-D-galactopyranoside was the best acceptor substrate among the monosaccharides, followed by methyl-β-D-galactopyranoside. For disaccharides, the truncated enzyme had slightly higher activity for the α-galactoside Gal-α-1,3-Gal-α-OMe, than for the β-galactosides Gal-β-1,3-Gal-β-OMe, lactose, Gal-β-1,3-GlcNAc-β-OMe and *N*-acetyllactosamine. 2′-Fucosyllactose (Fuc-α-1,2-Gal-β-1,4-Glc) was also a good acceptor. Because this enzyme was able to transfer NeuAc well to both α-galactoside and β-galactoside, this enzyme can be described as an α-/β-galactoside α2,3-sialyltransferase. Analysis of kinetic parameters supported the substrate preference of the enzyme (Table 6). The apparent $K_m$ value for methyl-α-D-galactopyranoside (0.18 mM) was almost the same as that for methyl-β-D-galactopyranoside (0.20 mM) (Table 6). Finally, the truncated enzyme had a lower apparent $K_m$ (0.14 mM) for lactose (Table 6).

For the α2,6-sialyltransferase, among the disaccharides, the β-galactosides such as Gal-β-1,3-Gal-β-OMe, lactose, Gal-β-1,3-GlcNAc-β-OMe and *N*-acetyllactosamine were the preferred acceptor substrates, whereas the α-galactoside of Gal-α-1,3-Gal-α-OMe was not a good acceptor. For monosaccharides, the truncated enzyme had high activity for methyl-β-D-galactopyranoside, but not for methyl-α-D-galactopyranoside. Fuc-α-1,2-Gal-β-1,4-Glc was as good an acceptor as lactose. Because this enzyme was able to transfer NeuAc well to β-galactoside, this enzyme can be described as a β-galactoside α2,3-sialyltransferase. Analysis of kinetic parameters supported the substrate preference of the enzyme (Table 6). The apparent $K_m$ value for lactose (31 mM) was approximately a third that for methyl-β-D-galactopyranoside (99 mM) (Table 6).

*Mass Spectrometry and NMR Spectroscopy*—After the reaction of each truncated recombinant enzyme with benzyl-β-D-galactopyranoside as the acceptor substrate, the reaction product was analysed by ESI-MS and $^1H$ and $^{13}C$ NMR spectroscopy. The $[M\text{-}H]^-$ ion peak for both enzymatic product was at 559.9 in the mass spectrum, indicating that the product was sialylbenzyl-β-D-galactopyranoside (exact mass: 561.21). The chemical shift data for the reaction product are listed in Tables 7 and 8. Each value corresponded well to those reported for an α2,3-sialoside and an α2,6-sialoside (*35*). These results further confirmed that the truncated enzymes were an α2,3-sialyltransferase and an α2,6-sialyltransferase.

Table 4. **Monosaccharide and oligosaccharide acceptor specificities of the truncated recombinant α/β-galactoside α2,3-sialyltransferase and β-galactoside α2,6-sialyltransferase.**

| Acceptor | α2,3-sialyltransferase | | α2,6-sialyltransferase | |
|---|---|---|---|---|
| | NeuAc transferred (nmol/min) | Relativeactivity (%)[a] | NeuAc transferred (nmol/min) | Relativeactivity (%) |
| Methyl-α-D-galactopyranoside | 8.49[b] | 823 | 0.04[c] | 2 |
| Methyl-β-D-galactopyranoside | 3.54[b] | 343 | 1.11[d] | 59 |
| *N*-Acetyl-D-galactosamine | 0.78[d] | 76 | 0.23[d] | 12 |
| Gal-β-1,4-Glc (Lactose) | 1.03[d] | 100 | 1.88[d] | 100 |
| Gal-β-1,3-GlcNAc-β-OMe | 1.98[d] | 192 | 1.43[d] | 77 |
| Gal-β-1,4-GlcNAc (*N*-Acetyllactosamine) | 0.99[d] | 96 | 1.86[d] | 99 |

NeuAc transferred to acceptor was determined at a constant acceptor substrate concentration of 42 mM.
[a]Relative activity (%) is shown; lactose is 100% value. [b]Reaction time: 0.5 min. [c]Reaction time: 60 min. [d]Reaction time: 2 min.

Table 5. **Monosaccharide and oligosaccharide acceptor specificities of the truncated recombinant α/β-galactoside α2,3-sialyltransferase and β-galactoside α2,6-sialyltransferase.**

| Acceptor | α2,3-sialyltransferase | | α2,6-sialyltransferase | |
|---|---|---|---|---|
| | NeuAc transferred (nmol/min) | Relative activity (%)[a] | NeuAc transferred (nmol/min) | Relative activity (%) |
| Galα1,3-Galα-OMe | 8.80[b] | 528 | 0.02[c] | 2 |
| Galβ1,3-Galβ-OMe | 5.52[b] | 331 | 0.96[d] | 68 |
| Fucα1,2-Galβ1,4-Glc | 0.78[d] | 52 | 1.32[d] | 92 |
| Gal-β-1,4-Glc (Lactose) | 1.51[d] | 100 | 1.43[d] | 100 |

NeuAc transferred to acceptor was determined at a constant acceptor substrate concentration of 8.3 mM.
[a]Relative activity (%) is shown; lactose is 100% value. [b]Reaction time: 1 min. [c]Reaction time: 60 min. [d]Reaction time: 2 min.

Table 6. **Apparent kinetic parameters of the truncated recombinant α/β-galactoside α2,3-sialyltransferase and β-galactoside α2,6-sialyltransferase for acceptor substrates.**

| Compound[a] | α2,3-sialyltransferase | | α2,6-sialyltransferase | |
|---|---|---|---|---|
| | $K_m$ (mM) | $V_{max}$ (nmol/min) | $K_m$ (mM) | $V_{max}$ (nmol/min) |
| Lactose | 0.14 | 2.0 | 31 | 5.0 |
| Methyl-α-D-galactopyranoside | 0.18 | 1.8 | nt[b] | nt |
| Methyl-β-D-galactopyranoside | 0.20 | 1.6 | 99 | 0.86 |

[a]Kinetic parameters were determined at a constant CMP-NeuAc concentration of 4.2 mM. [b]nt, not tested.

Table 7. **$^1$H and $^{13}$C NMR chemical shifts of the reaction product catalyzed by truncated recombinant α/β-galactoside α2,3-sialyltransferase using benzyl-β-D-galactopyranoside as an acceptor.[a]**

| Sugar unit-position | Proton atom (p.p.m.) | Carbon atom (p.p.m.) |
|---|---|---|
| Gal-OBn-1 | 4.56 | 102.00 |
| Gal-OBn-2 | 3.58 | 69.36 |
| Gal-OBn-3 | 4.08 | 76.72 |
| Gal-OBn-4 | 3.96 | 67.80 |
| Gal-OBn-5 | 3.68 | 75.30 |
| Gal-OBn-6 | 3.76 | 61.35 |
| –O–$CH_2$-ph | 7.45 | 128.94 |
| –O–$CH_2$-ph | 4.77 | 71.92 |
| –O–$CH_2$-ph | 4.96 | 71.92 |
| NeuAc-1 | – | 177.45 |
| NeuAc-2 | – | 99.96 |
| NeuAc-3ax | 1.79 | 39.93 |
| NeuAc-3eq | 2.76 | 39.93 |
| NeuAc-4 | 3.68 | 68.71 |
| NeuAc-5 | 3.84 | 52.10 |
| NeuAc-6 | 3.62 | 73.24 |
| NeuAc-7 | 3.59 | 68.81 |
| NeuAc-8 | 3.86 | 72.00 |
| NeuAc-9 | 3.64 | 63.06 |
| NeuAc-9 | 3.86 | 63.06 |
| NeuAc- N–C=O | – | 175.22 |
| NeuAc–$CH_3$ | 2.03 | 22.37 |

[a]$^1$H NMR, The spectrum was recorded at 298 K, using hydroxyl group of $H_2O$ as an internal reference (chemical shift, 4.75 p.p.m.); $^{13}$C NMR, The spectrum was recorded at 298 K, using dioxan in $D_2O$ as an external reference (chemical shift, 67.4 p.p.m.).

Table 8. **$^1$H and $^{13}$C NMR chemical shifts of the reaction product catalysed by truncated recombinant β-galactoside α2,6-sialyltransferase using benzyl-β-D-galactopyranoside as an acceptor.[a]**

| Sugar unit-position | Proton atom (p.p.m.) | Carbon atom (p.p.m.) |
|---|---|---|
| Gal-OBn-1 | 4.46 | 102.68 |
| Gal-OBn-2 | 3.52 | 71.48 |
| Gal-OBn-3 | 3.60 | 73.35 |
| Gal-OBn-4 | 3.94 | 69.33 |
| Gal-OBn-5 | 3.76 | 74.08 |
| Gal-OBn-6 | 3.64 | 64.03 |
| Gal-OBn-6 | 3.95 | 64.03 |
| –O–$CH_2$-ph | 7.47 | 129.51 |
| –O–$CH_2$-ph | 7.47 | 137.36 |
| –O–$CH_2$-ph | 4.75 | 72.45 |
| –O–$CH_2$-ph | 4.95 | 72.45 |
| NeuAc-1 | – | 174.15 |
| NeuAc-2 | – | 101.19 |
| NeuAc-3ax | 1.72 | 41.00 |
| NeuAc-3eq | 2.75 | 41.00 |
| NeuAc-4 | 3.71 | 69.02 |
| NeuAc-5 | 3.84 | 52.63 |
| NeuAc-6 | 3.71 | 73.41 |
| NeuAc-7 | 3.60 | 69.00 |
| NeuAc-8 | 3.90 | 72.45 |
| NeuAc-9 | 3.65 | 63.39 |
| NeuAc-9 | 3.89 | 63.39 |
| NeuAc- N–C=O | – | 175.70 |
| NeuAc-$CH_3$ | 2.04 | 22.75 |

[a]$^1$H NMR, The spectrum was recorded at 298 K, using hydroxyl group of $H_2O$ as an internal reference (chemical shift, 4.75 p.p.m.); $^{13}$C NMR, The spectrum was recorded at 298 K, using dioxan in $D_2O$ as an external reference (chemical shift, 67.4 p.p.m.).

## DISCUSSION

We isolated a novel organism, the marine bacterium *Photobacterium* sp. JT-ISH-224 from the gut of a Japanese barracuda (*S. pinguis*) that produced two sialyltransferases, α-/β-galactoside α2,3-sialyltransferase and β-galactoside α2,6-sialyltransferase. This bacterium is the first identified as having α2,3- and α2,6-sialyltransferases although two bacteria, *Campylobacter jejuni* (*16*, *36*) and *H. influenzae* (*37*), have a different pair of

sialyltransferases (α2,3-sialyltransferase and α2,3-/α2,8-bifunctional sialyltransferase). Also, a few bacteria have a bifunctional sialyltransferase, for example, *E. coli* (α2,8-/α2,9-sialyltransferase) (*38*), *N. meningitidis* (α2,3-/α2,6-sialyltransferase) (*19*) and *P. multocida* (α2,3-/α2,6-sialyltransferase) (*6*). Because of the diverse abilities for producing sialyltransferases bacterial pathogens and symbionts may have a strategy for evolving to carry multiple sialyltransferases and/or multifunctional sialyltransferases to synthesize complex specific oligosaccharide chains that mimic surface carbohydrates of host cells to escape immune systems and/or to adhere to the surface of the host cell. Therefore, we consider that bacterial pathogens and symbionts have the potential of harbouring sialyltransferases.

Mammalian sialyltransferases are specific for the terminal sugar, for the sugar next to the terminal galactose, and for the linkage between the terminal galactose and the second sugar. Bacterial sialyltransferases have a similar specificity, but the specificity is lower than that of mammalian sialyltransferases. In this respect, the specificity of α2,3- and α2,6-sialyltransferases from *Photobacterium* sp. JT-ISH-224 is qualitatively similar to those from *P. phosphoreum* JT-ISH-467 and *P. damselae* JT0160, respectively. There are some quantitative differences between the α2,3-sialyltransferase from *Photobacterium* sp. JT-ISH-224 and that from *P. phosphoreum* JT-ISH-467 (*21*). The apparent $K_m$ for lactose (0.14 mM) was approximately one-twelfth lower than the apparent $K_m$ of the enzyme from *P. phosphoreum* JT-ISH-467 (apparent $K_m$ of 1.7 mM), and the apparent $K_m$ values for monosaccharides (0.18 mM for methyl-α-D-galactopyranoside and 0.20 mM for methyl-β-D-galactopyranoside) were one-third or approximately one-seventh lower than that for the enzyme from *P. phosphoreum* JT-ISH-467 (0.54 mM for methyl-α-D-galactopyranoside and 1.3 mM for methyl-β-D-galactopyranoside, respectively). Therefore, the enzyme may be an advantage for the addition of NeuAc at the low concentration of the acceptor substrates. In addition, the apparent $K_m$ values of the enzyme from *Photobacterium* sp. JT-ISH-224 for lactose, methyl-α-D-galactopyranoside and methyl-β-D-galactopyranoside were almost the same, and the enzyme is not specific for the sugar next to the terminal galactose, nor for the linkage between the terminal galactose and the second sugar. There are also some quantitative differences between the α2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 and that from *P. damselae* JT0160. The sialyltransferase from *P. damselae* had high affinity to lactose (apparent $K_m$ of 6.82 mM) and was even active for methyl-β-D-galactopyranoside, with a higher apparent $K_m$ of 174 mM (*20*), whereas its apparent $K_m$ (31 mM) for lactose was higher than the apparent $K_m$ of the enzyme from *P. damselae*. The apparent $K_m$ for methyl-β-D-galactopyranoside, however, was lower (99 mM). Therefore, the enzyme from *Photobacterium* sp. JT-ISH-224 recognizes not only the non-reducing terminal galactose unit, but also the reducing terminal unit to which the galactose is attached, although the specificity of discrimination between lactose and methyl-β-D-galactopyranoside is lower than the enzyme from *P. damselae*.

The deduced amino acid sequences from the genes coding for the α2,3-sialyltransferase and α2,6-sialyltransferase in *Photobacterium* sp. JT-ISH-224 have high homology (92 and 54% identity) with the sialyltransferases from *P. phosphoreum* (*21*) and *P. damselae* (*25*), respectively, classified with GT family 80 in the CAZy data base. Phylogenic analysis revealed that the three groups (α2,3-sialyltransferases in *Photobacterium* sp., α2,6-sialyltransferases in *Photobacterium* sp. and sialyltransferases in Pasteurellaceae) might have arisen from a common ancestor although the chronological order of the splitting of these three groups is unclear (Fig. 2). Also, the discovery that this bacterium has two sialyltransferases suggests that the two sialyltransferases from *Photobacterium* were probably derived paralogously from a common ancestral gene in the *Photobacterium*.

From the deduced primary sequences of the cloned α2,3-sialyltransferase and α2,6-sialyltransferase, we can predict a conservative and short $NH_2$ terminal cytoplasmic domain of two positively charged amino acids ($Lys^6$ and $Lys^7$) and one positively charged amino acid ($Lys^2$), respectively (*39*), a signal-membrane anchor domain containing the lipobox motif (*33*), and a large catalytic domain on the COOH terminal side, but the enzyme does not contain the α-helix structure that is homologous to the Pho U protein for putatively anchoring the cell membrane as does the *P. damselae* sialyltransferase at the COOH terminal region (*25*). The primary structure of the large catalytic domain appears to be similar to the sialyltransferase from *P. multocida* because the nine amino acids interacting with CMP as a component of the donor substrate (*34*) correspond very closely to the two sialyltransferases from *Photobacterium* sp. JT-ISH-224 (Fig. 1), and because three of five amino acids ($His^{112}$, $Asp^{141}$ and $Trp^{270}$) that interact with lactose as a component of the acceptor substrate (*40*) correspond to those of the α2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 and one of these five ($Asp^{141}$) corresponds to that in the α2,3-sialyltransferases from *Photobacterium* sp. JT-ISH-224 (Fig. 1). Because the amino acid sequences of two sialyltransferases from *Photobacterium* sp. JT-ISH-224 contain the lipobox motif, it is possible that the proteins are modified with lipids and transported within the membrane in the periplasmic space as reported for *E. coli* (*39*).

The ultimate objective of our research is to supply a stable sialyltransferase having characteristic acceptor substrate specificity to synthesize various sialosides and to modify various glycoconjugates. We confirmed that the α2,3-sialyltransferase has very broad acceptor specificity and has low sialidase activity. The α2,6-sialyltransferase has a different acceptor specificity than the α2,6-sialyltransferase from *P. damselae* and is highly specific and productive. In this respect, these sialyltransferases hold high promise as good tools for producing various sialosides to modify glycoconjugates.

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Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under accession numbers AB293984, AB293985 and AB293986.

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