Purification, Cloning, and Expression of an α/β-Galactoside α-2,3-Sialyltransferase from a Luminous Marine Bacterium, Photobacterium phosphoreum*

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A novel sialyltransferase, α/β-galactoside α-2,3-sialyltransferase, was purified from the cell lysate of a luminous marine bacterium, Photobacterium phosphoreum JT-ISH-467, isolated from the Japanese common squid (Todarodes pacificus). The gene encoding the enzyme was cloned from the genomic library of the bacterium using probes derived from the NH2-terminal and internal amino acid sequences. An open reading frame of 409 amino acids was identified, and the sequence had 32% identity with that of β-galactoside α-2,6-sialyltransferase in Photobacterium damselae JT0160. DNA fragments that encoded the full-length protein and a protein that lacked the sequence between the 2nd and 24th residues at the NH2 terminus were amplified by polymerase chain reactions and cloned into an expression vector. The full-length and truncated proteins were expressed in Escherichia coli, producing active enzymes of 0.25 and 305 milliunits, respectively, per milliliter of the medium in the lysate of E. coli. The truncated enzyme was much more soluble without detergent than the full-length enzyme. The enzyme catalyzed the transfer of CMP-α-methyl-galactopyranoside to disaccharides, such as lactose and N-acetylactosamine, with much lower apparent Km than the full-length enzyme. Thus, this sialyltransferase is unique and should be very useful for achieving high productivity in E. coli with a wide substrate range.

The oligosaccharide chains in glycoconjugates, such as glycoproteins and glycolipids, have important roles in key biological processes, such as inflammatory and immunological responses, cell-cell recognition, cancer metastasis, and fertilization in diverse eukaryotes (1, 2). The oligosaccharide moieties are synthesized by a series of glycosyltransferases in the cells (3, 4). In humans, 110 genes encoding glycosyltransferases have been cloned so far (5). Prokaryotes also have clusters of genes encoding glycosyltransferases (3, 4). In humans, 110 genes encoding glycosyltransferases have been cloned so far (5). Prokaryotes also have clusters of genes encoding glycosyltransferases and related enzymes for synthesis of various oligosaccharide chains (6, 7). Several bacterial pathogens and symbionts have evolved specific oligosaccharide chains mimicking surface carbohydrates of host cells, which are crucial for self/nonself recognition, to escape immune systems (1, 4, 8).

Basic studies on the structure and function of the oligosaccharide chains in glycoconjugates have contributed to the development of a number of pharmaceutical products. For example, the addition of two more sialylated glycans to the hormone erythropoietin, which has three sialylated glycans in the native form, resulted in a considerably longer half-life of the hormone in blood compared with the native protein (9). Fully synthetic oligosaccharide vaccines have been examined to control infections by bacteria, such as Hemophilus influenzae type b (10), viruses such as human immunodeficiency viruses (11), and the protist that causes malaria (11). Carbohydrate microarrays have also been used to investigate the carbohydrate-binding specificities of bacteria, to detect pathogens in diagnosis, and to screen anti-adhesion therapeutics (11, 12).

Terminal sugars play key roles in the functions of glycochains, and sialic acid is often found as an α-sialoside at noreducing termini of carbohydrate chains in glycoconjugates. Thus, sialylated glycans are of particular interest in functional glycomics, and an ample supply of various sialylated glycans and glycoconjugates is essential for basic studies and the subsequent development of industrial products.

Sialylated glycans may be synthesized either chemically or enzymatically. Because the chemical addition of sialic acid to oligosaccharides requires complicated multistep processes and the stereospecific synthesis of α-sialosides is difficult, enzymatic methods are generally preferred. Although many sialyltransferases have been cloned from mammals to date, it is not easy to produce these enzymes in large quantity, because mammalian glycosyltransferases are rarely expressed as active enzymes in Escherichia coli. In general, bacterial enzymes are much more productive in E. coli expression systems, and bacterial glycosyltransferases have acceptor-substrate specificity much broader than mammalian glycosyltransferases (13–15). Thus, bacterial enzymes are expected to serve as indispensable tools in the preparation and modification of sialylated glycans (16–19).

To meet this demand, diverse bacterial sialyltransferases are needed with respect to substrate specificity, solubility, pH, and temperature range for activity, productivity, and other enzymatic properties. At present, sialyltransferases are classified into five families, which differ in their structurally related cata-
lytic and carbohydrate-binding modules, in the CAZy (carbohydrate-active enzymes) data base (available on the World Wide Web) (20). Four of these families consist of bacterial enzymes. However, the extent of 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.

We have been screening a large number of marine bacteria for novel glycosyltransferase activities and hereby report on the purification, characterization, and gene cloning of a novel enzyme, α/β-galactoside α-2,3-sialyltransferase, from a luminous marine bacterium, Photobacterium phosphoreum JT-ISH-467, which was isolated from the surface of Japanese common squid (Todarodes pacificus).

**EXPERIMENTAL PROCEDURES**

**Screening of Bacteria**—Samples of seawater, sand, mud, small animals, and seaweed were collected from coasts of various locations in Japan. Bacteria that grew on marine agar 2216 (BD Biosciences) or nutrient agar (BD Biosciences) that was supplemented with 2% NaCl, and 0.03% Triton X-100. The reaction was carried out at 25 °C for 2 h.

**Sialyltransferase Assay**—The 30-μl reaction mixture consisted of a sample of enzyme, 120 mM lactose, 2.3 mM CMP-NeuAc (Nakarai), 4620 Bq ofCMP-[4,5,6,7,8,9-14C]NeuAc, 25 mM sodium cacodylate buffer (pH 6.0) that contained 0.2% Triton X-100 and 0.03% Triton X-100, and 0.03% Triton X-100, and lysed by sonication on ice. Cellular debris was removed by centrifugation at 100,000×g for 10 min.

**Sialylation of Pyridylaminated Lactose**—The 20-μl reaction mixture was composed of a sample of sialyltransferase, 1.25 mM 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 mixture was then applied to a PALPAK type R analytical column (0.46 × 25 cm; Takara Bio) that was equilibrated with 100 mM acetic acid-triethylamine buffer (pH 5.0) that contained 0.15% n-butanol in a high pressure liquid chromatograph (LC10A; Shimadzu). 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 (excitation, 320 nm; emission, 400 nm). The retention times for pyridylaminated lactose, pyridylaminated 6′-sialyllactose (prepared using α-2,6-sialyltransferase from P. damselae JT0160 (21)), and pyridylaminated 3′-sialyllactose (PA-Sugar Chains 029; Takara Bio) were 4.00, 4.39, and 5.40 min, respectively.

**Sialidase Activity**—The 15-μl reaction mixture consisted of a sample of enzyme, 1.7 μM pyridylaminated 3′-sialylactose, 15 mM bis-Tris buffer (pH 6.0), and 0.1% Triton X-100 and was incubated at 25 °C. The mixture was analyzed using a PALPAK type R analytical column under the same conditions described earlier.

**Purification of α-2,3-Sialyltransferase**—Strain JT-ISH-467 was shaken in 10 ml of marine broth 2216 at 25 °C for 24 h. Then 300 ml of a medium that consisted of 37.4 g/liter marine broth 2216, 20 g/liter peptone (BD Biosciences), and 4 g/liter yeast extract (BD Biosciences) was inoculated with the seed culture and incubated for 2 h on a rotary shaker at 200 rpm. Bacteria were harvested by centrifugation to yield a pellet. The pellet was then suspended in 16 ml of 20 mM bis-Tris buffer (pH 6.0) that contained 0.3% Triton X-100 (Buffer A) per g 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 HiPrep 16/10 DEAE FF column (1.6 × 10 cm; Amersham Biosciences) equilibrated with Buffer A in a protein liquid chromatography system (AKTA; Amersham Biosciences). After the column was washed extensively with Buffer A, 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 to three volumes with 20 mM potassium phosphate buffer (pH 6.0) that contained 0.3% Triton X-100 (Buffer B), and applied to a hydroxyapatite column of Bio-scale CHT20-I (1.5 × 11.3 cm; Bio-Rad) equilibrated with Buffer B. After the column was washed 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 to two volumes with Buffer A, and loaded on a column of Mono Q 10/100 GL (1 × 10 cm; Amersham Biosciences) equilibrated with Buffer A. After the column was washed with Buffer A, the enzyme was eluted with a linear gradient of NaCl from 0 to 1 M in Buffer A. The active fractions were pooled, diluted to three volumes with 20 mM potassium phosphate buffer (pH 7.0) that contained 0.3% Triton X-100 (Buffer C), and applied to a column of Mono Q 10/100 GL equilibrated with Buffer C. After the column was washed with Buffer C, the enzyme was eluted with a linear gradient from 0 to 1 M NaCl in Buffer C. The active fractions were pooled and loaded on HiLoad 16/60 Superdex 200 pg (1.6 × 60 cm; Amersham Biosciences) equilibrated with Buffer C that contained 0.2 M NaCl.


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The active frations were collected. All of the chromatographic purification steps were performed at 7 °C.

**Amino Acid Sequencing**—The NH₂ terminus of the purified protein was sequenced with a Procise 494 cLC protein sequencing system (Applied Biosystems). For the analysis of internal sequences, 10 μg of a purified protein was digested with lysyl endopeptidase in 100 mM Tris-Cl buffer (pH 8.5) for 20 h at 35 °C. The digest was applied to a Symmetry C18 3.5-μm analytical column (0.1 × 15 cm; Waters) and eluted with a continuous linear gradient of acetonitrile from 2 to 90% in 0.1% trifluoroacetic acid. The peptides from the three strongest peaks were analyzed by the protein sequencer.

**Matrix-assisted Laser Desorption Time-of-flight Mass Spectrometry (MALDI-TOFMS)—**MALDI-TOF MS was performed for 10–20 pmol of a purified protein with an AXIMA-CFR MALDI-TOF mass spectrometer (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. For peptide mass fingerprinting of the protein, ~10 pmol of a purified protein was digested with trypsin in 100 mM Tris-Cl buffer (pH 8.0) for 20 h at 35 °C. The digest was applied to a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems) operated in the reflector mode, using 2.5-dihydroxybenzoic acid as the matrix.

**Amplification of the Partial Gene**—Unless mentioned otherwise, the methods used for molecular cloning and DNA manipulation were those described by Maniatis et al. (22). Genomic DNA was isolated from bacteria using Genomic-tip 100/G (Qiagen). The PCR mixture of 50 μl consisted of 5 pmol of each primer sequence (Table 1), 10 nmol of dNTPs, 2.5 units of Ex TaqDNA polymerase, and 5 μl of 10× Ex Taq buffer according to the manufacturer's instructions (Takara Bio). 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 40 cycles, and further incubated at 72 °C for 6 min in a GeneAmp PCR System (model 9600; PerkinElmer Life Sciences). The PCR product was cloned into pCR2.1 TOPO vector (Invitrogen). The DNA sequence was determined by an ABI PRISM model 310 genetic analyzer (PerkinElmer Life Sciences).

**Genomic Library**—The genomic DNA was partially digested with Sau3AI. The fragments between 9 and 16 kb and the BamHI arms of PPST1, PPST2, and PPST3 (Table 1), were designed to create PciI, Ncol, and BamHI restriction sites, respectively, at the ends of the PCR products. The entire gene was synthesized by 15 cycles of PCR using PPSTF1 and PPSTR1 under the same conditions described earlier except for the cycle numbers. The PCR product was digested with PciI and BamHI and cloned between the Ncol and BamHI sites of expression vector pTrc99A (Amer sham Biosciences) to give plasmid pPP-ST3-FL. A truncated gene was amplified using PPSTF2 and PPSTR1 by the same method, digested with Ncol and BamHI, and cloned between the Ncol and BamHI sites of pTrc99A to give pPP-ST3-A2–24.

**16 S rRNA Gene**—The 16 S rRNA gene was amplified using primers 27f and 1525r (Table 1) (23) from the genomic DNA by PCR under the same conditions described, except for annealing at 55 °C, elongation for 1 min, and 30 cycles.

**Recombinant α-2,3-Sialyltransferases—**E. coli TB1 that harbored pPP-ST3-FL was shaken in 3 ml of Luria-Bertani (LB) broth (Type Miller; BD Biosciences) that contained 100 μg/ml ampicillin at 30 °C for 16 h. Then 300 ml of the same medium containing 1 ml isopropyl 1-thio-β-D-galactopyranoside was inoculated with the seed culture and incubated for 16 h. The full-length recombinant enzyme was prepared according to the procedures used to purify the protein from P. phosphoreum, except that HiPrep 16/10 DEAE FF was replaced with HiLoad 26/10 Q Sepharose HP (2.6 × 10 cm; Amersham Biosciences). The truncated recombinant enzyme was also purified, using the same method as for the full-length recombinant enzyme, from E. coli TB1 that harbored the pPP-ST3-A2–24.

**pH and Temperature Profiles**—For the pH profile, the reaction in the native and full-length recombinant sialyltransferase assay was carried out at 25 °C for 10 min, whereas that for the truncated recombinant enzyme was at 30 °C for 10 min 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 buffer (pH 8.0–9.0), 100 mM CHES buffer (pH 9.0–10.0), or 100 mM CAPS 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 no more than 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), methyl-β-D-galactopyranoside (Sigma), methyl-α-D-glucopyranoside (Sigma), methyl-

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**TABLE 1**

| Primer | Sequence* |
|--------|-----------|
| PPST1  | 5′-AYT WSN GAY WSN AAR CAY AAY AA-3′ |
| PPST2  | 5′-CAT RTT RDP WNO RTC DAT GKT-3′ |
| PPST4  | 5′-AGG GAA ATA CAT GGT COT TTT TG |
| PPSTF1 | 5′-AAW GGA ATA CAT GGT COT TTT TG |
| PPSTF2 | 5′-CCT AGA ATG GAT CCT CAT TGC AAA |
| PPSTR1 | 5′-TTT AGA ATG GAT CCT CAT TGC AAA |
| 27f    | 5′-AGA GGG GTA GCC TGC GTC AAT-3′ |
| 1525r  | 5′-AAA GGA GGT GAT CCT CAT TGC GTC AAT-3′ |

* Y, T or C; W, T or A; S, C or G; R, A or G; N, A, T, or G.  
* Primers were designed to create restriction sites (underlines) and start and stop codons (boldface letters).
**TABLE 2**

| Chromatography step | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) | Purification fold |
|---------------------|-------------|--------------------|-----------------------|----------------------------|-----------|-----------------|
| Crude extract       | 3155        | 6159               | 8.4                   | 1.4                        | 100       | 1.0             |
| DEAE                | 410         | 932                | 3.1                   | 3.4                        | 37        | 2.5             |
| Hydroxyapatite      | 264         | 153                | 1.3                   | 8.2                        | 15        | 6.0             |
| Mono Q (pH 6)       | 12          | 24                 | 0.96                  | 39                         | 11        | 29              |
| Mono Q (pH 7)       | 1.5         | 1.7                | 0.52                  | 315                        | 6.2       | 229             |
| Superdex 200        | 1.5         | 0.2                | 0.10                  | 457                        | 1.2       | 333             |

*One unit of activity was defined as the amount of enzyme that transferred 1 μmol of NeuAc per min to lactose in the standard assay described under “Experimental Procedures,” and the reaction was performed at pH 5 and 25 °C.*

| Sample | Volume (ml) | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) | Purification fold |
|--------|-------------|--------------------|-----------------------|----------------------------|-----------|-----------------|
| Crude extract | 3155 | 6159 | 8.4 | 1.4 | 100 | 1.0 |
| DEAE   | 410         | 932                | 3.1                   | 3.4                        | 37        | 2.5             |
| Hydroxyapatite | 264 | 153 | 1.3 | 8.2 | 15 | 6.0 |
| Mono Q (pH 6) | 12 | 24 | 0.96 | 39 | 11 | 29 |
| Mono Q (pH 7) | 1.5 | 1.7 | 0.52 | 315 | 6.2 | 229 |
| Superdex 200 | 1.5 | 0.2 | 0.10 | 457 | 1.2 | 333 |

**RESULTS**

Isolation and Identification of Marine Bacteria Producing α-2,3-Sialyltransferase—More than 3000 isolates of bacteria were examined for sialyltransferase activity, and 10 of these tested positive in the enzymatic assay. The 10 isolates were tested for activity in the transfer of sialic acid to pyridylaminated lactose; four isolates had α-2,3-sialyltransferase activity, yielding pyridylaminated 3′-sialyllactose. We focused on one of the α-2,3-sialyltransferase producers, JT-ISH-467, isolated from the outer skin of Japanese common squid (T. pacificus) in this study, because JT-ISH-467 had the highest α-2,3-sialyltransferase activity.

The 16 S rRNA gene of JT-ISH-467 was partially sequenced (1500 bp, accession number AB293983) and was identical to that of P. phosphoreum ATCC11040 (the type strain) (accession number X74687). Optimal bacterial growth was observed at 15–25 °C on nutrient agar supplemented with 1–3% NaCl. The bacterium grew to some extent at 4 °C but not at 30 °C and was luminescent at 4–17.5 °C. Because these characteristics corresponded well to the type strain of P. phosphoreum (25), we concluded that JT-ISH-467 was a strain of P. phosphoreum.

**Purification of α-2,3-Sialyltransferases**—Total sialyltransferase activity from JT-ISH-467 cultured in marine broth 2216 at 25 °C for 24 h reached 1.4 milliunits/ml culture medium. Although only 7.5% of the activity was evident without detergent, 54% of the activity was present with 0.3% Triton X-100. The enzyme was purified 333-fold through the five chromatographic steps in the presence of 0.3% Triton X-100 with a yield of 1.2% (Table 2). The purified enzyme migrated as a single polypeptide with a molecular mass of ~40,000 Da in electrophoresis.
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Cloning of α-2,3-Sialyltransferase Gene—Amplification of partial gene fragments was attempted from the genome of JT-ISH-467 using pairs of degenerated primers, which were designed based on the amino acid sequences. Primer pair PPST1 and PPST2 (Table 1) yielded a PCR product of 929 bp, and of ~5000 clones from a genomic library constructed from JT-ISH-467 screened with the cloned PCR product as a probe, 24 positive clones were identified. After restriction analysis and Southern hybridization, a 4.6-kb HindIII fragment that hybridized to the probe was subcloned from one of the clones. An open reading frame of 1230 bp (accession number AB293982), which fully contained the 929-bp sequence, was found in the clone and encoded a protein of 409 amino acids (protein ID BAF63530) (supplemental Fig. 1) with a calculated molecular mass of 46,758 Da. The sequence between residues 23 and 32 of the reading frame was identical to the sequence between residues 2 and 11 of the NH₂-terminus of the purified protein (supplemental Fig. 1). The NH₂-terminal residue of the purified protein, which could not be determined by peptide sequencing, was deduced to be cysteine (supplemental Fig. 1). There were stretches identical to the three internal peptide sequences described earlier in the reading frame (supplemental Fig. 1). We concluded that the cloned gene coded for the α-2,3-sialyltrasferase from P. phosphoreum JT-ISH-467.

The first 21 amino acid residues appeared to be removed from the mature protein. The processed enzyme predicted from the DNA sequence was composed of 388 amino acids with a calculated mass of 44,308 Da, although the molecular mass of the enzyme was 45,026 Da (accuracy 1000 ppm) in the MALDI-TOF MS analysis as described earlier. There was a difference in molecular mass of ~700 Da. This sequence constituted a positively charged amino-terminal region that contained two positively charged amino acids (Lys⁶ and Lys⁷) and a hydrophobic region (Ile²⁸–Ile³⁸) (supplemental Fig. 1). The Leu¹⁹–Cys²² sequence was identical to the lipobox sequence (supplemental Fig. 1) (26). The protein was predicted to be a lipoprotein (signal peptidase II-cleaved proteins) via a method to predict lipoprotein signal peptides in Gram-negative eubacteria using the hidden Markov model, LipoP (available on the World Wide Web) (27). The ScanProsite tool (available on the World Wide Web) (28) also predicted that the protein was a lipoprotein. These results indicated that the first 22 residues acted as a putative signal peptide and suggested that the protein was a lipoprotein.

A BLAST search in the public data base did not detect any matches with the nucleotide sequence of the gene but showed that the amino acid sequence of the sialyltrasferase from JT-ISH-467 had 32, 29, and 22% identity to GT family 80 enzymes, β-galactoside α-2,6-sialyltrasferase (protein ID BAA25316) in P. damselae JT0160 (29), multifunctional sialyltrasferase (protein ID AAY90601) in Pasteurella multocida subsp. multocida Pm70 (15), and a hypothetical protein (protein ID AAP95068) in Haemophilus ducreyi 35000HP, respectively (supplemental Fig. 1). The residues that had been identified as sites for binding to CMP in the sialyltrasferase from P. multocida (30) appeared to be conserved mostly in the sialyltrasferase from JT-ISH-467 (supplemental Fig. 1). On the other hand, the protein had no homology with sialyltrasferases of the other families. Thus, this enzyme can be classified in GT family 80 in the CAZy data base.

Peptide Mass Fingerprinting—Because no significant peaks at a molecular mass of 640–670 were found, the predicted NH₂-terminus trypsin-digested peptide fragment, Cys²²–Lys²⁷ ([M + H]⁺, 653.26) was not present. In the molecular mass range of 1320–1420, five significant peaks (m/z = 1348.65, 1363.65, 1370.79, 1385.67, and 1407.68) were detected. The masses of m/z = 1363.65, 1385.67, and 1407.68 were assigned to trypsin-digested deduced peptide fragment, Ser¹⁰⁰–Lys¹¹¹ ([M + H]⁺, 1363.71, δ 44 ppm; [M + Na]⁺, 1385.70, δ 22 ppm; and [M + 2Na]⁺, 1407.68, δ 0 ppm), respectively. Also, the masses of m/z = 1363.65, 1385.67, and 1407.68 were assigned to trypsin-digested peptide fragments, Phe²⁵⁰–Lys⁵⁰⁰ ([M + H]⁺, 1363.63, δ 15 ppm; [M + Na]⁺, 1385.61, δ 43 ppm; and [M + 2Na]⁺,
mass of the enzyme was sialyltransferase from grown in an 8.1-liter culture. The enzyme was purified 18-fold (total activity, 1489 units) was prepared from 63 g of wet cells with or without the detergent, respectively. Crude extract (reached 0.25 milliunits/ml culture medium after 16 h, and 72 or 51% of the activity was present with or without 0.3% Triton X-100 detergent, respectively. Crude extract (total activity, 10 units) was prepared from 67 g of wet cells grown in a 10.8-liter culture. The enzyme was purified 101-fold through four chromatographic steps: Q Sepharose, hydroxyapatite, Mono Q, and Superdex in the presence of the detergent with a yield of 3.3%. The specific activity of the purified enzyme was 0.3 units/mg.

Total sialyltransferase activity from E. coli with pPP-ST3-FL, induced with isopropyl 1-thio-β-D-galactopyranoside at 30 °C, reached 0.25 milliunits/ml culture medium after 16 h, and 50 or 23% of the activity was present with or without 0.3% Triton X-100 detergent, respectively. Crude extract (total activity, 10 units) was prepared from 67 g of wet cells grown in a 10.8-liter culture. The enzyme was purified 101-fold through four chromatographic steps: Q Sepharose, hydroxyapatite, Mono Q, and Superdex in the presence of the detergent with a yield of 3.3%. The specific activity of the purified enzyme was 0.3 units/mg. The enzyme migrated as a single polypeptide with a molecular mass of ~40,000 Da in electrophoresis under denaturing conditions. The molecular mass of the enzyme was 45,023 Da (accuracy 1000 ppm) in MALDI-TOF MS, although the enzyme predicted from the DNA sequence was composed of 409 amino acids with a calculated mass of 46,758 Da. The NH2 terminus of the enzyme appeared to be blocked.

Total sialyltransferase activity from E. coli with pPP-ST3-Δ2–24 assayed in the same way reached 305 milliunits/ml culture medium after 16 h, and 72 or 51% of the activity was present with or without the detergent, respectively. Crude extract (total activity, 1489 units) was prepared from 67 g of wet cells grown in an 10.8-liter culture. The enzyme was purified 101-fold through four chromatographic steps: Q Sepharose, hydroxyapatite, Mono Q, and Superdex in the presence of the detergent with a yield of 3.3%. The specific activity of the purified enzyme was 0.3 units/mg. The enzyme migrated as a single polypeptide with a molecular mass of ~40,000 Da in electrophoresis under denaturing conditions. The molecular mass of the enzyme was 45,023 Da (accuracy 1000 ppm) in MALDI-TOF MS, although the enzyme predicted from the DNA sequence was composed of 409 amino acids with a calculated mass of 46,758 Da. The NH2 terminus of the enzyme appeared to be blocked.

**Expression of α-2,3-Sialyltransferase in E. coli**—Expression cassettes of the full-length gene for sialyltransferase in pPP-ST3-FL and of the truncated form, which lacks the NH2-terminal hydrophobic region in pPP-ST3-Δ2–24 were constructed and expressed in E. coli.

Total sialyltransferase activity from E. coli with pPP-ST3-FL, induced with isopropyl 1-thio-β-D-galactopyranoside at 30 °C, reached 0.25 milliunits/ml culture medium after 16 h, and 50 or 23% of the activity was present with or without 0.3% Triton X-100 detergent, respectively. Crude extract (total activity, 10 units) was prepared from 67 g of wet cells grown in a 10.8-liter culture. The enzyme was purified 101-fold through four chromatographic steps: Q Sepharose, hydroxyapatite, Mono Q, and Superdex in the presence of the detergent with a yield of 3.3%. The specific activity of the purified enzyme was 0.3 units/mg. The enzyme migrated as a single polypeptide with a molecular mass of ~40,000 Da in electrophoresis under denaturing conditions. The molecular mass of the enzyme was 45,023 Da (accuracy 1000 ppm) in MALDI-TOF MS, although the enzyme predicted from the DNA sequence was composed of 409 amino acids with a calculated mass of 46,758 Da. The NH2 terminus of the enzyme appeared to be blocked.

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**General Properties of α-2,3-Sialyltransferase**—The native enzyme from P. phosphoreum and recombinant enzymes were active between pH 5.0 and pH 11.0, similar to the range for the sialyltransferase from P. multocida (15). Temperatures between 20 and 25 °C and pH values between 5.0 and 5.5 were optimal for the native enzyme and the full-length recombinant, whereas temperatures between 25 and 30 °C and pH values between 5.5 and 6.0 were optimal for the truncated recombinant. The native and recombinant enzymes were twice as active in the presence of NaCl at 500, 750, and 1000 mM as in the absence of NaCl.

The truncated recombinant was examined for sialylation activity for pyridylaminated lactose, and production of pyridylaminated 3′-sialyllactose was confirmed. However, with prolonged incubation, the product from the reaction gradually decreased, indicating that the enzyme also had weak sialidase activity. Thus, the enzyme was assayed for degradation of pyridylaminated 3′-sialyllactose, and a low level of sialidase activity was indeed present in the enzyme.

Further analysis was made for the truncated enzyme, which had an NH2-terminal primary structure (MDSKHNNNSD) that was very similar to that of the native enzyme (XNSDKHNNNSD). The truncated enzyme was able to transfer NeuAc to all of the examined saccharides (Tables 3 and 4). Methyl-α-D-galactopyranoside was the most preferred acceptor substrate for the truncated enzyme among the monosaccharides, followed by methyl-β-D-galactopyranoside. For disaccharides, the truncated enzyme had slightly higher activity for α-galactoside, Gal-α,1,3-Gal-α-Ome, than for β-galactosides, Gal-β,1,3-Gal-β-Ome, lactose, Gal-β,1,3-GlcNAc-β-Ome, and N-acetyllactosamine. Analysis of kinetic parameters supported the substrate preference of the enzyme (Table 5). The apparent K<sub>m</sub> value for methyl-α-D-galactopyranoside (0.54 mM) was approximatively half that for methyl-β-D-galactopyranoside (1.3 mM) (Table 5). Finally, the truncated enzyme had a very low apparent K<sub>m</sub>, of 0.05 mM for CMP-NeuAc (Table 5).

**Mass and NMR Spectroscopy**—After the reaction of the truncated recombinant enzyme with benzyl-β-D-galactopyranoside as the acceptor substrate, the reaction product was analyzed by ESI-MS and 1H and 13C NMR spectroscopy. The [M − H]<sup>+</sup> ion peak of the enzymatic reaction product was at

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**TABLE 3**

| Acceptor | NeuAc transferred | Relative activity a |
|----------|-------------------|--------------------|
| Methyl-α-D-galactopyranoside | 10.4<sup>b</sup> | 452 |
| Methyl-β-D-galactopyranoside | 5.8<sup>b</sup> | 251 |
| Methyl-α-D-galactofuranoside | 1.1<sup>b</sup> | 48 |
| Methyl-β-D-glucopyranoside | 0.8<sup>a</sup> | 33 |
| Methyl-α-D-mannopyranoside | 1.4<sup>c</sup> | 60 |
| Methyl-β-D-mannopyranoside | 1.5<sup>c</sup> | 63 |
| Methyl-α-D-fucopyranoside | 1.1<sup>c</sup> | 49 |
| Methyl-β-D-fucopyranoside | 1.0<sup>c</sup> | 42 |
| N-Acetyl-D-galactosamine | 1.0<sup>c</sup> | 43 |
| N-Acetyl-D-glucosamine | 0.9<sup>c</sup> | 40 |
| Gal-β,1,4-Glc (lactose) | 2.3<sup>c</sup> | 100 |
| Gal-β,1,3-GlcNAc-β-Ome | 3.0<sup>c</sup> | 129 |
| Gal-β,1,4-GlcNAc (N-acetyllactosamine) | 2.4<sup>c</sup> | 103 |

<sup>a</sup> Relative activity (%) is shown; lactose is 100% value.
<sup>b</sup> Reaction time was 0.5 min.
<sup>c</sup> Reaction time was 2 min.

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**TABLE 4**

| Acceptor | NeuAc transferred | Relative activity a |
|----------|-------------------|--------------------|
| Gal-α,1,3-Gala-OMe | 7.2<sup>a</sup> | 438 |
| Gal-β,1,3-Gal-β-OMe | 5.2<sup>a</sup> | 316 |
| Fuc-α,1,2-Galβ1,4-Glc | 0.8<sup>a</sup> | 46 |
| Lactose | 1.6<sup>a</sup> | 100 |

<sup>a</sup> Relative activity (%) is shown; lactose is 100% value.
<sup>b</sup> Reaction time: 1 min.
<sup>c</sup> Reaction time 2 min.
α/β-Galactoside α-2,3-Sialyltrasferase from P. phosphoreum

TABLE 5
Apparent kinetic parameters of truncated recombinant α/β-galactoside α-2,3-sialyltrasferase for donor and acceptor substrates

| Compound            | $K_{\text{m}}$  | $V_{\text{max}}$ |
|---------------------|----------------|-----------------|
| Lactose             | 1.7 ± 0.2      | 0.37 ± 0.01     |
| N-Acetyllactosamine | 2.5 ± 0.3      | 0.40 ± 0.02     |
| Methyl-α-β-galactopyranoside | 0.54 ± 0.05 | 0.34 ± 0.03     |
| Methyl-β-α-galactopyranoside | 1.3 ± 0.1 | 0.34 ± 0.02     |

a Kinetic parameters were determined at a constant lactose concentration of 120 mM.
b Kinetic parameters were determined at a constant CMP-NeuAc concentration of 4.2 mM.

559.9 in the mass spectrum, indicating that the product was sialylbenzyl-β-n-galactopyranoside, which was 561.21 in exact mass. The chemical shift data measured for the reaction product are listed in supplemental Table 1. These values corresponded well to those reported for an α-2,3-sialoside (31). These results further confirmed that the truncated enzyme was an α-2,3-sialyltrasferase.

DISCUSSION

We found a novel sialyltrasferase from a bacterium, JT-ISH-467, which was identified as a strain of P. phosphoreum, a bioluminescent light-organ symbiont of several deep sea fishes (32). P. phosphoreum often causes spoilage in stored squids (33). This is the first sialyltrasferase isolated from symbiotic bacteria and characterized in detail. The gene for the enzyme was cloned, sequenced, and expressed in E. coli, producing highly active recombinant enzymes. The α-2,3-sialyltrasferase activity was identified in assays using pyridylamminated lactose and confirmed by mass and NMR spectroscopy of the enzymatic reaction products. Because this enzyme was able to efficiently transfer NeuAc to both α-galactoside and β-galactoside, this enzyme can be described as an α/β-galactoside α-2,3-sialyltrasferase.

The nucleotide sequence of the sialyltrasferase does not have any homologous sequences in the public data base, but the deduced amino acid sequence of the enzyme had homology (20–35% identity) to the sialyltrasferases classified in the GT family 80 in the CAZy data base. Photobacterium belongs to Vibrionaceae, and the expressers of sialyltrasferases in GT family 80 have so far been restricted to species of Vibrionaceae (29) and Pasteurellaceae (15) in γ-proteobacteria, whereas the expressers of enzymes in the other GT families were found widely in β-, γ-, and ε-proteobacteria and in Gram-positive bacteria. Thus, GT family 80 enzymes probably originated from a common ancestral gene that had evolved after the γ-proteobacteria had separated from other groups of proteobacteria. The sialyltrasferases in GT family 80 may be one of the latest expressers of enzymes in the family 80 in the CAZy data base.

The NH$_2$-terminal amino acid sequence (Met$^1$–Cys$^{22}$) (supplemental Fig. 1) of the sialyltrasferase from JT-ISH-467 appeared to have the composition of other bacterial prelipoprotein signal peptides; it is composed of an n-region, an h-region, and a c-region, which are conserved consensus sequences (34–38). The n-region is a positively charged amino-terminal region that contains the positively charged amino acids lysine and/or arginine in the first 5–7 residues; the h-region consists of 7–22 hydrophobic amino acids; and the c-region has a characteristically more polar carboxyl-terminal cleavage region that consists of four amino acids, a so-called lipobox (the amino acid distribution in the lipobox is shown on the World Wide Web (26)) (27, 35–37). The sialyltrasferase from P. damselae also had a lipobox (supplemental Fig. 1) and, in the COOH terminus, an α-helix putative cell membrane-anchoring structure, which was homologous to Pho U protein (29) and absent from the enzyme from JT-ISH-467. The multifunctional sialyltrasferase from P. multocida Pm70 did not have a lipobox (supplemental Fig. 1); a different signal peptide must be present, because the 25 amino acids in the NH$_2$ terminus of a recombinant sialyltrasferase from P. multocida were specifically cleaved in E. coli (15). Thus, various structures for signal functions and cellular membrane association may be present among the enzymes in GT family 80, suggesting that the sialyltrasferases in the family are diverse with respect to cellular locations and biological roles in bacteria. On the other hand, catalytic domains appear to be similar to each other among GT family 80 enzymes. Large catalytic domains were found in the COOH-terminal sides of many of the enzymes in the group, and the sites for binding to CMP (30), a key component of the donor substrate for sialic acid, were well conserved (supplemental Fig. 1). Binding sites for acceptor substrates have not been elucidated yet for GT family 80 enzymes. Further studies of the sialyltrasferase from JT-ISH-467, such as examination with X-ray crystallography in the presence and absence of acceptor substrates, may give an important clue.

Lipoproteins had not been found previously among glycosyltrasferases, but several lines of evidence suggest that the sialyltrasferase from JT-ISH-467 is a lipoprotein. As described earlier, the NH$_2$-terminal amino acid sequence of the sialyltrasferase appeared to fit the pattern of having the three conserved consensus sequences of prelipoprotein signal peptides. The presence of the lipobox of the enzyme is an especially typical characteristic of a lipoprotein. The molecular mass differed by ~700 Da between the calculation from the deduced amino acids and the measurements by MALDI-TOF MS of both the native enzyme and the full-length recombinant, whereas the molecular mass did not differ much between the calculated and measured mass of the truncated recombinant, which lacked the lipobox sequence. The discrepancy can be explained by modification of the cysteine with a lipid. Further analysis of the native enzyme by peptide mass fingerprinting gave further evidence that the NH$_2$-terminal peptides that were digested with a peptidase, trypsin, appeared to be modified with a mass of ~700 Da. Moreover, the function predictor programs, LipoP and ScanProsite, for uncharacterized proteins, each predicted that the enzyme was a lipoprotein (27, 28). If so, the protein might be retained in the periplasmic space through the association of the lipid with the membrane of the bacteria. This hypothesis is consistent with the observation that the protein was quite insoluble without detergent and that the enzyme was more active in a high concentration of NaCl, such as found in the periplasm, which is naturally filled with seawater. The fact that removal of the lipobox sequence resulted in higher solubility of the recombinant protein suggests that the lipobox sequence is essential for the membrane association of the enzyme.


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