Cloning and Expression of a Marine Bacterial β-Galactoside α2,6-Sialyltransferase Gene from Photobacterium damsela JT0160

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Sialyltransferase 0160, a bacterial sialyltransferase which catalyzes the incorporation of NeuAc from CMP-NeuAc into the galactose residue of the carbohydrate chain at position 6, is produced by Photobacterium damsela JT0160. The gene coding for sialyltransferase 0160 (bst) was cloned, sequenced, and expressed in Escherichia coli. The sialyltransferase 0160 gene contains an open reading frame of 2,028 base pairs encoding a protein of 675 amino acid residues. The deduced amino acid sequence of sialyltransferase 0160 did not contain the sialylmotif and had no significant similarity to mammalian sialyltransferases. Crude extracts of cultured E. coli MV1184 cells carrying an expression plasmid for the sialyltransferase 0160 gene showed sialyltransferase activity, which was identified as β-galactoside α2,6-sialyltransferase activity by enzymatic reaction product analysis. In addition, when mutant genes, lacking 3'-coding regions for COOH-terminal portions of the protein, which are thought to form α-helix structures, were expressed in E. coli MV1184, soluble-form enzymes were obtained. This implies that the COOH-terminal portion of sialyltransferase 0160 is required for membrane binding.

Key words: bacterial sialyltransferase, cloning, Photobacterium damsela JT0160, α2,6-sialyltransferase.
Molecular Cloning and DNA Sequencing—Most of the methods used for molecular cloning were based on those of Maniatis et al. (19). P. damsela JT0160 cells were prepared as described previously (17). Genomic DNA of P. damsela JT0160 was isolated by the method of Saito and Miura (20). Colony hybridization was performed by the method of Hanahan and Meselson (21). DNA sequencing was carried out by the dideoxy chain termination method (22). Restriction enzymes and other nucleic acid-modifying enzymes were purchased from Takara; HindIII linker and XbaI linker from New England Biolabs. Plasmid pUC19, HindIII linker and XbaI linker were purchased from Takara; M13mp18 and M13mp19 were from Bio-Rad.

Organism and Culture Conditions—P. damsela JT0160 was maintained at 18°C on the marine broth 2216-agar plates (37.4 g of marine broth, 1.5 g of agar, 1 liter of distilled water, pH 7.6). E. coli strain XL-1 Blue MRA (P2) [Δ(mcrA) 183 Δ(mrcCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 gyrA96 relA1 lacP2 lysogen] was used as a host for plasmids and grown in L-broth medium at either 30°C or 40°C. E. coli XL-1 Blue MRA (P2) [4(mcrA) 183 4(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 gyrA96 relA1 lacP2 lysogen] was used as a host for phages and grown on an L-broth medium supplemented with 0.2% maltose and 10 mM MgSO4. The cells were recovered by centrifugation and resuspended in 10 mM MgSO4, E. coli strain MV1184 [ara Δ(lac-pro) strA thi φ 80dlacIZ ΔM15 Δ(srl-recA)306::Tn10(Tet)]; F' :traD 36 proABlacI 2Z ΔM15] was used as a host for plasmids and grown in L-broth medium at either 37 or 30°C. Ampicillin (100 μg/ml) and IPTG (0.02 mM) were added when needed.

Determination of Partial Amino Acid Sequence of Sialyltransferase 0160—Sialyltransferase 0160 was purified from P. damsela JT0160 by the method of Yamamoto et al. (17). To identify the NH2-terminal amino acid sequence, the purified sialyltransferase 0160 was sequenced with a protein sequencer (Applied Biosystems model 473A) according to the method of Matsudaira (23). The partial amino acid sequences were determined as follows. Ten micrograms of purified sialyltransferase 0160 was digested with trypsin (5 U) for 24 h at 37°C. The reaction mixture was subjected to HPLC (μRPC C2/C18 SC 2.1/10, Pharmacia) and eluted with a continuous linear gradient of 2 to 100% acetonitrile. The amino acid sequences of some peaks were analyzed on a protein sequencer.

Expression of Sialyltransferase 0160 Gene—E. coli MV1184 carrying expression plasmids was cultured in medium containing IPTG. Culture was done at 30°C on a rotary shaker (150 rpm) for 24 h, then the cells were centrifuged (20 min at 7,000×g), and suspended in 50 ml of 20 mM sodium cacodylate buffer (pH 6.0). The suspension was sonicated and centrifuged at 100,500×g for 1 h. The enzyme assays were carried out using the supernatant obtained and sonicated cells as enzyme sources.

Purification of Recombinant Sialyltransferase 0160—The purification of the recombinant sialyltransferase 0160 was carried out according to method reported previously (17). In brief, cells were harvested from the culture by centrifugation (6,000×g, 20 min). They were suspended in 20 mM sodium cacodylate buffer (pH 6.0) and sonicated. The sonicated solution was centrifuged (105,000×g, 1 h) and the supernatant obtained was used as crude enzyme solution. From this, recombinant sialyltransferase 0160 was purified by using a combination of anion exchange chromatography, hydroxyapatite chromatography, gel filtration chromatography, and affinity chromatography.

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

The identification of enzymatic reaction products was performed by HPLC. The enzymatic reactions were carried out using pyridylaminated lactose as an acceptor substrate. After the reaction, each reaction mixture was analyzed by HPLC using a Takara PALPAK type R (0.46 × 25 cm) analytical column. The reaction mixture was applied to the column equilibrated with 100 mM acetic acid-triethylamine buffer (pH 5.2) containing 0.15% n-butanol. Pyridylaminated carbohydrate chains (non-reacted acceptor substrate and product) were eluted using n-butanol in the same buffer. The concentration of n-butanol was increased linearly from 0.15 to 0.5% (0–35 min). Pyridylaminated carbohydrate chains were detected by fluorescence measurement (Ex: 320 nm; Em: 400 nm). The column temperature was 40°C, and the flow rate was 1 ml/min (24).

Site-Directed Mutagenesis—The oligodeoxynucleotides for site-directed mutagenesis were synthesized with an Applied Biosystems 381A DNA synthesizer. To introduce a stop codon at 539 L and 498 D, primer MTY01 [5'-CAAA ACAATTACTGATTAATAGTGAATTGGCGATGTGGCA-3'] was used as mutagenic primer. To introduce an EcoRI site just upstream of methionine, primer MTY03 [5' -TTTTTATGTGAATGTGGAATTCATGA-3'] was used as mutagenic primer. Site-directed mutagenesis were performed with phage
M13mp18 and M13mp19, using a MUTA-GENE in vitro mutagenesis kit (Bio-Rad), according to Kunkel (25). The entire regions of the DNA fragments were sequenced to confirm that only the expected mutation had occurred.

Other Methods—SDS-electrophoresis was done by the method described by Laemmli (37). The nucleotide and amino acid sequences were evaluated using the DNASIS computer program developed by Hitachi Software Engineering (Yokohama). Accessed data bases were Genbank (National Institute of Health) and EMBL.

RESULTS

Cloning and Sequencing of Sialyltransferase 0160 Gene—First, the genomic DNA of P. damsela JT0160 was digested with HindIII and Southern hybridization were carried out using primer HTY01. The resulting 2.8 kbp fragment was hybridized. To obtain recombinant plasmids containing this fragment, the 2.8 kbp fragment derived from HindIII-digested genomic DNA was ligated into the HindIII site of pUC19. No recombinant plasmid which hybridized with HTY01 was obtained.

Then, the genomic DNA was partially digested with Sau3A1 and the digested fragment was ligated with T4 DNA ligase into the BamHI site of lambda phage DASH II (Stratagene) at 4°C, 16 h. Recombinant lambda DNAs were packaged with Gigapack II packaging extract (Stratagene). Packaged recombinant lambda phages DASH II were used to infect E. coli XL-1 Blue MRA (P2) at 37°C for 15 min, and infected cells were spread on L-broth medium agar plates in a solution of 0.7% agarose. From these plates, recombinant lambda phages were recovered. The recovered lambda phages were used as a genomic DNA library.

The genomic library were distributed on L-broth medium agar plates at the 1,000 plaques per plate and plaque hybridization was carried out using the primer HTY01. From one of the positive clones (lambda-7), lambda DNA was purified using lambda-prep (Promega). The obtained lambda DNA was digested with HindIII. The digest was ligated into the HindIII site of pUC19 and then transformed into E. coli MV1184 cells. To obtain positive clones, colony hybridization was carried out using the primer HTY01. From the positive clones, amplified recombinant plasmid DNAs were recovered by standard methods (19). All these plasmids contained a 1.6 kbp fragment in the HindIII site of the sialyltransferase 0160 gene. Therefore, it was clear that the nucleotide sequence of this reading frame is that of the gene encoding sialyltransferase 0160 (Fig. 2). The hydrophathy profile of the deduced amino acid sequence of sialyltransferase 0160, obtained according to Kyte and Doolittle (26), is shown in Fig. 3. The first 15 amino acid residues constitute a hydrophobic region. Moreover, after Met, two 2 positively charged amino acid residues (Lys) were present. These results indicated that this region may function as a signal sequence translocating the protein across the cytoplasmic membrane (39).

The deduced amino acid sequence of the primary translation product of sialyltransferase 0160 gene had no sialyl-motif and showed no apparent sequence similarity with any other sialyltransferase of mammalian origin. But, the COOH-terminal regions of the primary translation product showed high homology with the phosphate transport system regulatory protein (PhoU protein) of E. coli (27) (Fig. 4). The secondary structure analysis of the primary translation product showed that the COOH-terminal regions of sialyltransferase 0160 may formed a α-helix structures (amino acid residue 543 to 561, amino acid residue 569 to 588), and hydrophobic amino acid residues were arranged in one direction.

In the sequence upstream of the initiation codon, three regions homologous to the ribosome-binding and promoter sites of E. coli were found. A Shine-Dalgaro site was located 41 bases upstream from the initiation codon (28). Two promoter-like sequences, GATATT and ATGACA, were present. These regions constitute a potential promoter. In the 3'-noncoding regions of the sialyltransferase gene, a potential translational termination sequence which could form a stem-and-loop structure (nucleotides 2054 to 2081) followed by a thymine-rich sequence. This structure may protect the end of the mRNA from the attack of single-strand-specific 3'-
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Fig. 2. Nucleotide sequence and the deduced amino acid sequence of the sialyltransferase 0160 gene. The numbering of nucleotides starts at the 5' terminus of the bst gene, and that of amino acids at the NH₂ terminus of mature sialyltransferase 0160. Typical expression signals, a promoter sequence (−35 and −10 regions), a Shine-Dalgarno sequence (SD), and putative translational termination sequence are underlined. The termination codon is indicated by an asterisk. Double-underlined amino acid sequences were obtained from sialyltransferase 0160 degraded with trypsin.

Fig. 3. Hydropathy profile of sialyltransferase 0160. Hydropathy was calculated according to Kyte and Doolittle (26). The portions above the horizontal line correspond to hydrophobic regions.

Shine-Dalgarno sequence (SD), and putative translational terminatin sequence are underlined. The termination codon is indicated by an asterisk. Double-underlined amino acid sequences were obtained from sialyltransferase 0160 degraded with trypsin.

RNases (31) in addition to terminating transcription.

Construction of Expression Plasmid—The pAQN plasmid DNA which was constructed to express the aqualysin I gene (aqul) (32) was used as the expression plasmid for bst. This plasmid DNA contains lacIq from pMJR1560, on and Amp' from pUC18, and aquI gene between the EcoRI site just downstream of the tac promoter and the HindIII site just upstream of the rrnBT1T2 terminator. Ten unique restriction sites including EcoRI and HindIII exist between the EcoRI site and the HindIII site. The construction of the expression plasmid for bst, pEBST was carried out as follows.

(1) Modification of pAQN: The restriction enzyme sites of pAQN are in the sequence EcoRI, BglII, XbaI, and HindIII from just downstream of the tac promoter. First, pAQN was digested with HindIII and XbaI, followed by treatment with DNA polymerase Klenow fragment, and
ligated. By this treatment, the HindIII site was excised from pAQN and the XbaI site was recovered (pAQN₆XH). Second, pAQN₆XH was digested with BglII, followed by Klenow fragment treatment, and ligated with HindIII linker. By this treatment, the BglII site was substituted with a HindIII site (pAQN₆EHX).

(2) Modification of pBSTC and ligation of the inserted DNA fragment into pAQN₆EHX: The 1.2-kbp HindIII-digested fragment derived from pBSTC was ligated into the HindIII site of the multiple cloning sites of M13mp18. The plasmid DNA with the portion encoding the COOH-terminal region of sialyltransferase 0160 on the EcoRI site side in the multiple cloning sites of M13mp18 was selected, and named pMBSTC. This was digested with HpaI, followed by treatment with Klenow fragment, and ligated with XbaI linker. The resulting HpaI site was substituted with an XbaI site (pMBSTC₆XH). The HindIII-XbaI fragment of pMBSTC₆XH was ligated into the HindIII-XbaI site of pAQN₆EHX (designated pEBSTC).

(3) Modification of pBSTN and construction of expression plasmid: A 1.6-kbp HindIII fragment derived from pBSTN was ligated into the HindIII site of the multiple cloning sites of M13mp19. The plasmid DNA with the DNA region encoding the OH-terminal amino acid sequence of sialyltransferase 0160 on the EcoRI site side in the multiple cloning sites of M13mp19 was selected, and named pMBSTN. To introduce an EcoRI site just upstream of methionine, site-directed mutagenesis was carried out using primer MTY03, and the resulting plasmid was designated pMBSTNₑ. The EcoRI-HindIII fragment of pMBSTNₑ was ligated into the EcoRI-HindIII site of pEBSTC. The resulting plasmid was designated pEBST (Fig. 5). The bst gene could be inserted only into the plasmid containing the lacᵉ gene.

Expression of Sialyltransferase 0160 in E. coli—E. coli MV1184 carrying pEBST was cultured. After culture, cells were collected by centrifugation and suspended in 20 mM sodium cacodylate buffer (pH 6.0). The suspension was sonicated, and the enzyme activity of the sonicated solution was measured. Sialyltransferase activity was observed in it (Table 1).

| Table 1. The enzyme activity of each cell sonicate and its supernatant after ultracentrifugation. | The enzyme activity was measured by the method described under "MATERIALS AND METHODS.” The enzyme activity was expressed as total activity of the cells obtained from 1 liter of culture broth. |
| Plasmid transformed into E. coli MV1184 | Enzyme activity of cell sonicate | Enzyme activity of supernatant of ultracentrifugation |
| pEBST | 26.2 U/liter | N.D. |
| pEBSTₑ | 56.6 U/liter | 37.3 U/liter |
| pEBSTₑ₁₇₈ | 224.5 U/liter | 121.3 U/liter |

Stem Tase 0160 and PhoU protein. The amino acid sequences from the NH₂-terminal residue of the proteins. StTase: The amino acid sequence of sialyltransferase 0160 [amino acid residue, 498 (D) to 675 (D)]. PhoU protein: The amino acid sequence of the phosphate transport system regulatory protein [amino acid residue, 56 (D) to 239 (R)]. Consensus amino acids are shown at the bottom of each line.

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Fig. 6. SDS-PAGE of purified recombinant sialytransferase. The electrophoresis was carried out on a 10% polyacrylamide gel at pH 8.4 in Tris-glycine buffer. Proteins were stained with Coomassie Brilliant Blue R-250. Phosphorylase B (105 kDa), bovine serum albumin (82 kDa), ovalbumin (49 kDa), carbonic anhydrase (33.3 kDa), and soybean trypsin inhibitor (20.6 kDa) were used as standards for molecular mass determination. Lane 1, standard proteins; molecular masses are indicated. Lane 2, recombinant sialytransferase.

Discussion

In order to clarify the primary structure of sialytransferase 0160, we have cloned, sequenced, and expressed in E. coli the gene coding for sialytransferase 0160 (bst) from a genomic DNA of P. damsela JT0160. In 1987, Weinstein et al. first cloned a cDNA encoding a Galβ1, 4GlcNAc α2,6-sialytransferase using polyclonal antibodies raised against the purified enzyme (33). Up to present, 12 sialytransferase genes have been cloned. Comparison of their primary amino acid sequences has revealed two conserved regions, named “sialyl motif L” and “sialyl motif S,” in the catalytic domain (34). The “sialyl motif L” has been demonstrated to be involved in binding of the common donor substrate of sialytransferase, CMP-NeuAc (16). On the other hand, the bacterial α2,3-sialytransferase genes from Neisseria meningitidis and Neisseria gonorrhoeae were cloned and expressed in E. coli. The genes encoding bacterial α2,3-sialytransferases showed no similarity to those of mammalian sialytransferases (35). The results of homology search showed that our sialytransferase 0160 has no homologous regions to the cloned sialytransferases, even though sialytransferase 0160 shares some features of donor substrate and acceptor substrate specificity. These results indicated that sialytransferase 0160 may have acquired its activity through an evolutionary process different from that of the mammalian and N. meningitidis and N. gonorrhoeae enzymes.

The structure of sialytransferase 0160 seems to be quite different from those of known sialytransferases. The deduced primary sequences of the cloned sialytransferases predicted a short NH₂-terminal cytoplasmic domain, a signal-membrane anchor domain, a stem region, and a large catalytic domain on the COOH-terminal side of the mature proteins. However, sialytransferase 0160 seems to bind to the membrane through α-helix structures at COOH-terminal regions, like pyruvate oxidase purified from E. coli (36). Soluble-form COOH-terminal deletion mutants were obtained (E. coli. MV1184 carrying pEBST₂137 and pEBST₂178, Table I). From these results, it was predicted that (1) the catalytic domain of sialytransferase 0160 may lie in the NH₂-terminal side of the protein, (2) α-helix structures which may be formed in the COOH-terminal regions of the enzyme seem to be involved in membrane binding.

The primary translation product of the bst gene between the initiation and termination codons is predicted to be composed of 675 amino acid residues with a calculated weight of 76.5 kDa. The purified sialytransferase 0160 from P. damsela JT0160 has an NH₂-terminal amino acid sequence identical to the sequence from the 16th to 31st amino acids of the primary translation product of the bst gene. The first 15 amino acid residues constitute a hydrophobic region that probably acts as a signal peptide. Therefore, the mature enzyme is composed of 660 amino acid residues with a calculated weight of 74.8 kDa. However, the purified enzyme from P. damsela JT0160 showed a molecular weight of 61 kDa on SDS-PAGE analysis and 64 kDa on gel-filtration chromatography (17). Thus, the mature enzyme may be formed by processing at the NH₂- and/or COOH-terminal ends. On the assumption that processing at the COOH-terminal side occurred at the end of the α-helix structures, the predicted mature enzyme would be composed of 573 amino acid residues with a calculated weight of 64.5 kDa. This calculated weight is consistent with the values obtained by SDS-PAGE and gel-filtration chromatography. Analysis of the COOH-terminal amino acid sequence of sialytransferase 0160 is in progress.

P. damsela JT0160 is Gram-negative bacterium, and there is a candidate signal peptide in the NH₂-terminal region of the deduced amino acid sequence of sialytransferase 0160. Thus, sialytransferase 0160 may be translocated across the cytoplasmic membrane to the periplasm. It may be involved in the biosynthesis of glycoproteins which contain sialic acid. An outer membrane protein thought to be essential as an acceptor substrate of sialytransferase 0160 has been obtained from P. damsela JT0160 (data not shown). Further investigation of this outer membrane protein is in progress.

The 2.8-kbp fragment, derived from HindIII digestion of genomic DNA of P. damsela JT0160, which contained the bst gene, was not obtained using pUC19 as the host plasmid. The bst gene could be obtained only by using plasmid containing the lacI₃ gene as the host plasmid.
This indicates that bGal is gene is fatal to E. coli. The expression level of the recombinant gene was not optimized, but the levels of soluble-form enzyme activities we have produced are approximately 40 units/liter (pEBSTJ137) and 120 units/liter (pEBSTJ172) of culture (Table I). On the other hand, mammalian Gal1,4GlcNAc 2,6-sialyltransferase expressed in E. coli was found to accumulate in an insoluble form, and soluble enzyme was obtained by denaturation/renaturation steps (38). Moreover, the specific activity of purified recombinant sialyltransferase 0160 is slightly higher than that of the authentic enzyme. Thus, expression plasmid pEBST is a superior expression plasmid for production of a 2,6-sialyltransferase.

It is now possible to produce soluble-form a 2,6-sialyltransferase on a large scale. This should be valuable for large-scale enzymatic synthesis of a 2,6-sialylated oligosaccharides.

**REFERENCES**