J. Biochem. **123**, 94-100 (1998)

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

Takeshi Yamamoto,[1] Motoko Nakashizuka, and Ichiro Terada

Sea Water Science Research Laboratory, Japan Tobacco Inc., 4-13-20 Sakawa, Odawara, Kanagawa 256

Received for publication, July 30, 1997

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.

Sialic acid plays an important role in a variety of biological and physiological events, such as cell-cell recognition and virus infections (*1-4*). Usually, sialic acid is linked at the terminal position of carbohydrate chains of glycoproteins and glycolipids (*5*), and three linkage patterns, NeuAcα2-6Gal, NeuAcα2-3Gal, and NeuAcα2-6GalNAc, are commonly found in mammalian glycoproteins. These linkages are formed by specific sialyltransferases. Several sialyltransferases have been purified from mammalian sources (*6-9*). These enzymes, which usually catalyze the transfer of NeuAc from CMP-NeuAc, can be distinguished enzymatically by rigid specificity for the sequence of the acceptor oligosaccharide and the sugar to which it is attached.

The cloning of sialyltransferase genes of mammalian origin has revealed a conserved motif, the sialylmotif, in the catalytic domain of these enzymes (*10*). The presence of this sialylmotif has been used to aid cloning of this gene family, the sialyltransferase family (*11-15*). The sialylmotif has been demonstrated to be involved in binding of the common donor substrate CMP-NeuAc (*16*).

Recently, we reported the purification and some properties of sialyltransferase 0160, which catalyzes the incorporation of NeuAc from CMP-NeuAc into the galactose residue of the carbohydrate chain at position 6. This sialyltransferase 0160 was produced by a marine bacterium, *Photobacterium damsela* JT0160 (*17*). This enzyme shows unique acceptor specificity compared with mammalian enzymes. For example, in the case of sialyltransferase 0160, lactose has almost the same K_m value as *N*-acetyllactosaminide. Further, sialyltransferase 0160 catalyzes the transfer of a NeuAc to fucosyl- and sialyl-trisaccharides in very high yield (*18*). These features are quite different from those of rat liver β-galactoside α2,6-sialyltransferase. However, sialyltransferase 0160 shares some properties with mammalian sialyltransferases. For example, the donor substrate of sialyltransferase 0160 is CMP-NeuAc, the common donor substrate of mammalian sialyltransferases. Its acceptor substrate specificity is similar to that of rat liver α2,6-sialyltransferase, too. Methyl-β-D-galactopyranoside is the best acceptor substrate among the monosaccharides. Preference for the β-anomer is observed for assayed monosaccharides. Furthermore, lactose is a better substrate than methyl-β-D-galactopyranoside. These results indicated that sialyltransferase 0160 recognized not only the non-reducing terminal galactose unit, but also the reducing terminal unit to which galactose is linked. From the viewpoint of these donor and acceptor substrate specificity, the structures of the catalytic site and/or substrate binding site in the catalytic domain of sialyltransferase 0160 are expected to show some similarity to those of mammalian sialyltransferases. To clarify these points, we investigated the sialyltransferase 0160 gene.

[1] To whom correspondence should be addressed at the present address: Tobacco Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227. Phone: +81-45-973-5611, Fax: +81-45-973-6781

Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-perfomance liquid chromatography; *bst*, the gene coding for sialyltransferase 0160.

In this paper, we report (i) the nucleotide sequence of the gene for sialyltransferase 0160 and the deduced amino acid sequence, (ii) the production of enzymatically active, soluble-form sialyltransferase 0160 in *Escherichia coli*, and (iii) the putative membrane-binding site of sialyltransferase 0160.

MATERIALS AND METHODS

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 and New England Biolabs. Plasmid pUC19, *Hin*dIII linker and *Xba*I linker were purchased from Takara; M13mp18 and M13mp19 were from Bio-Rad.

Organism and Culture Conditions—*P. damsela* JT 0160 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*(*mcr*CB-*hsd*SMR-*mrr*) 173 *end*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *lac*P2 lysogen] was used as a host for lamda phages and grown on an L-broth medium agar plate (1% Tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar in distilled water) at 37°C. Liquid culture of *E. coli* XL-1 Blue MRA (P2) was carried out 5 ml of L-broth medium supplemented with 0.2% maltose and 10 mM $MgSO_4$. The cells were recovered by centrifugation and resuspended in 10 mM $MgSO_4$. *E. coli* strain MV1184 [*ara Δ*(*lac-pro*) *str*A *thi* ϕ 80d*lacIZ* *Δ*M15 *Δ*(*srl-rec*A)306::Tn10(Tet^r); F′:*traD* 36 *pro*AB*lacI* ^{q}Z *Δ* M15] 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 NH_2-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.

Based on the partial amino acid sequence, hybridization primer HTY01, [5′-GCIAAITIITIGCIGGIACIIIICCIGAI-GCICCIAA-3′] was synthesized. Here, 'I' indicated inosine.

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 collected by centrifugation (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-^{14}C]-NeuAc transferred from CMP-[4,5,6,7,8,9-^{14}C]-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-^{14}C]-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-^{14}C]-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-G-3′] and primer MTY02 [5′-TGTTCTGTTCTGGGCTT-AGTGATAAGATCTCTCGATGGAAGTTGCC-3′] were used as mutagenic primers, respectively. To introduce an *Eco*RI site just upstream of methionine, primer MTY03 [5′-TTTTTATGTGAATGTGGAATTCATGA-AGAAAATACTGA-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 *Hin*dIII 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 *Hin*dIII-digested genomic DNA was ligated into the *Hin*dIII site of pUC19. No recombinant plasmid which hybridized with HTY01 was obtained.

Then, the genomic DNA was partially digested with *Sau*3A1 and the digested fragment was ligated with T4 DNA ligase into the *Bam*H1 site of lamda phage DASH II (Stratagene) at 4°C, 16 h. Recombinant lamda DNAs were packaged with Gigapack II packaging extract (Stratagene). Packaged recombinant lamda 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 lamda phages were recovered. The recovered lamda 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 (lamda-7), lamda DNA was purified using lamda-prep (Promega). The obtained lamda DNA was digested with *Hin*dIII. The digest was ligated into the *Hin*dIII 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 *Hin*dIII site. These plasmids were named pBSTN. Next, using lamda-7 as the template, DNA sequencing was carried out using sequencing primer STY01 [5′-ATTTTTCAAGGGGCATCCTGCTGG-3′]. Based on the results of lamda-7 sequencing, hybridization primer HTY02 [5′-AAGATTTCATTTGAGGT-3′] was synthesized. Using primer HTY02, Southern hybridization were performed against *Hin*dIII-digested lamda-7 DNA. The resulting 1.2-kbp fragment was hybridized and this fragment was ligated into the *Hin*dIII site of pUC19. This plasmid was named pBSTC.

Nucleotide Sequence of the Gene for Sialyltransferase 0160—The restriction enzyme map of the 2.8-kbp *Hin*dIII fragment is presented in Fig. 1. Both strands of the fragment were sequenced. Figure 2 shows the nucleotide sequence of the DNA and the predicted amino acid sequence of the sialyltransferase 0160. A long open reading frame of 2,028 nucleotides was found. The amino acid sequence deduced from the nucleotide sequence of sialyltransferase 0160 had 675 amino acid residues with a calculated weight of 76.5 kDa (Fig. 2). The amino acid sequence from the 17th to the 31st amino acid of the primary translation product of this frame coincided with the NH_2-terminal amino acid sequence of sialyltransferase 0160. Furthermore, all of the partial amino acid sequences of sialyltransferase 0160 were coincided with the predicted sialyltransferase 0160 gene product. Therefore, it was clear that the nucleotide sequence of this reading frame is that of the gene encoding sialyltransferase 0160 (Fig. 2). The hydropathy 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 sialylmotif 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 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-Dalgarno site was located 41 bases upstream from the initiation codon (*28*). Two promoter-like sequences, GATATT and ATGACA, were observed at nucleotides −126 to −121 and −157 to −152, which were similar to the −10 and −35 regions of *E. coli*, respectively (*29, 30*). Therefore, 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′-

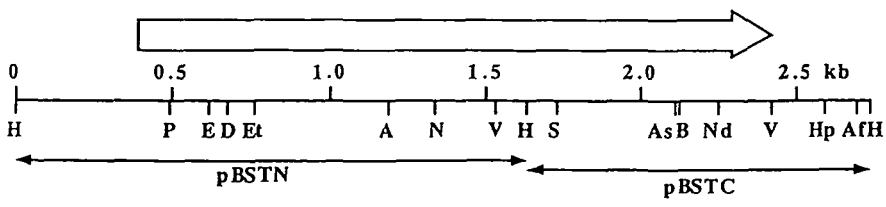


Fig. 1. **Restriction map and position of the cloned DNA fragment.** The restriction sites of plasmids are shown. The position of the open reading frame of the sialyltransferase 0160 gene is indicated by the open arrow. The DNA fragments contained by plasmids, pBSTN and pBSTC, are denoted by horizontal arrows.

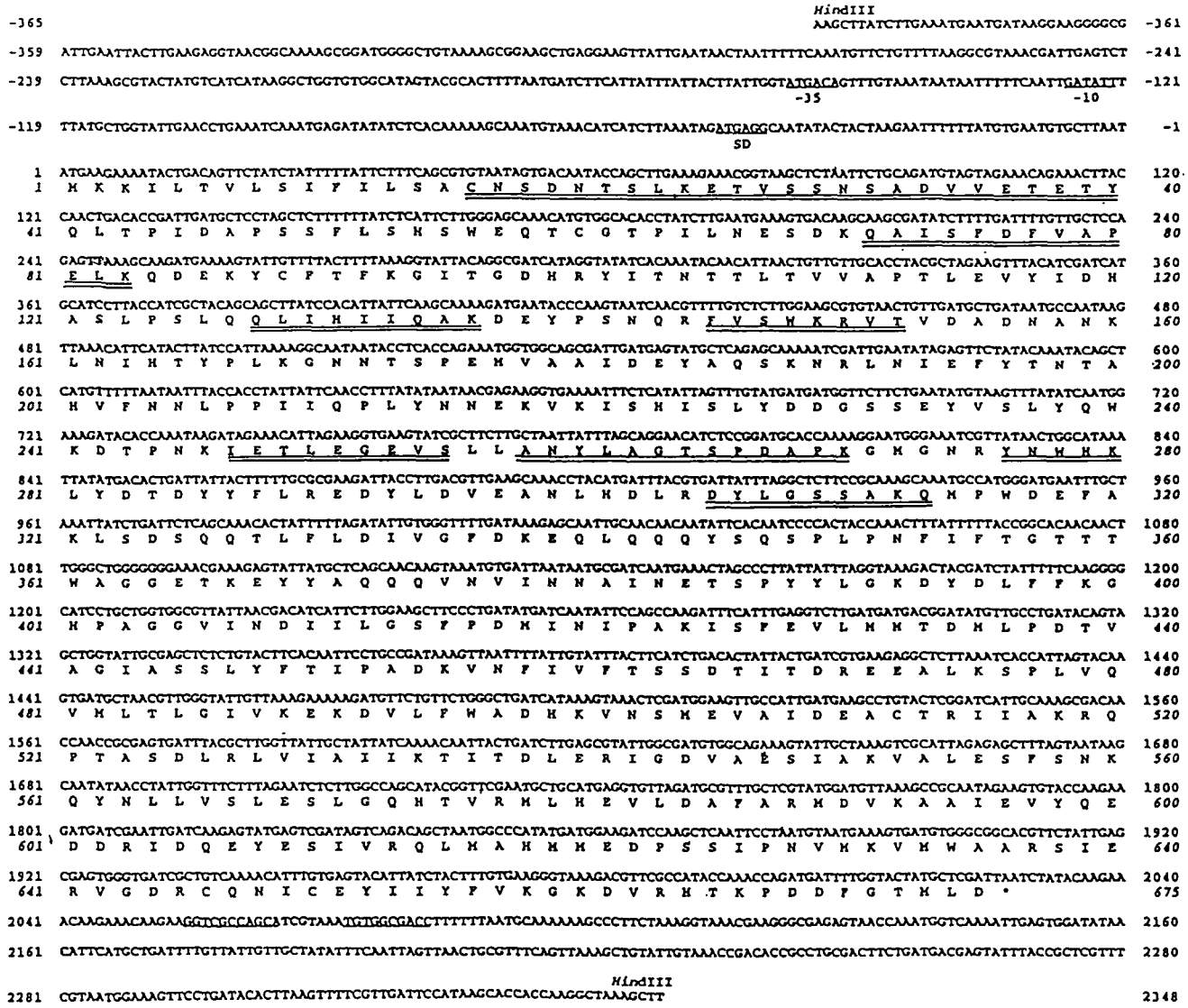


Fig. 2. **Nucleotide sequence and the deduced amino acid sequence of the sialyltransferase 0160 gene.** The numbering of nucleotides starts at the 5′ terminus of the *bst* gene, and that of amino acids at the NH_2 terminus of mature sialyltransferase 0160. Typical expression signals, a promoter sequence (−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.

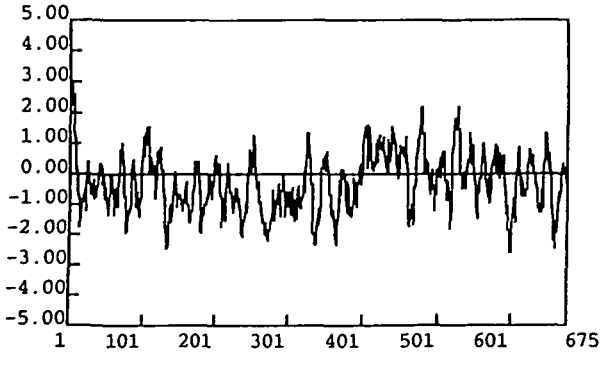


Fig. 3. **Hydropathy profile of sialyltransferase 0160.** Hydropathy was calculated according to Kyte and Doolittle (*26*). The portions above the horizontal line correspond to hydrophobic regions.

RNases (*31*) in addition to terminating transcription.

Construction of Expression Plasmid—The pAQN plasmid DNA which was constructed to express the aqualysin I gene (*aquI*) (*32*) was used as the expression plasmid for *bst*. This plasmid DNA contains *lacI*^{q} from pMJR1560, *ori* and *Amp*^{r} from pUC18, and *aquI* gene between the *Eco*RI site just downstream of the *tac* promoter and the *Hin*dIII site just upstream of the *rrnBT1T2* terminator. Ten unique restriction sites including *Eco*RI and *Hin*dIII exist between the *Eco*RI site and the *Hin*dIII 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 *Eco*RI, *Bgl*II, *Xba*I, and *Hin*dIII from just downstream of the *tac* promoter. First, pAQN was digested with *Hin*dIII and *Xba*I, followed by treatment with DNA polymerase Klenow fragment, and

ligated. By this treatment, the *Hin*dIII site was excised from pAQN and the *Xba*I site was recovered (pAQN⊿XH). Second, pAQN⊿XH was digested with *Bgl*II, followed by Klenow fragment treatment, and ligated with *Hin*dIII linker. By this treatment, the *Bgl*II site was substituted with a *Hin*dIII site (pAQN-EHX).

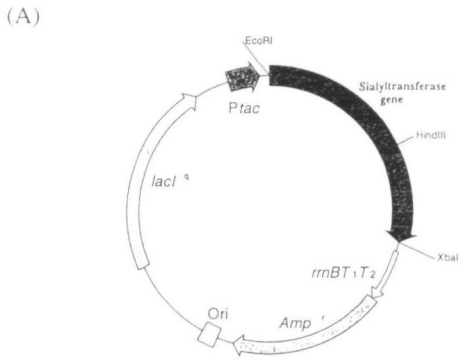
(2) Modification of pBSTC and ligation of the inserted DNA fragment into pAQN-EHX: The 1.2-kbp *Hin*dIII-digested fragment derived from pBSTC was ligated into the *Hin*dIII site of the multiple cloning sites of M13mp18. The plasmid DNA with the portion encoding the COOH-terminal region of sialyltransferase 0160 on the *Eco*RI site side in the multiple cloning sites of M13mp18 was selected, and named pMBSTC. This was digested with *Hpa*I, followed by treatment with Klenow fragment, and ligated with *Xba*I linker. The resulting *Hpa*I site was substituted with an *Xba*I site (pMBSTC-XH). The *Hin*dIII-*Xba*I fragment of pMBSTC-XH was ligated into the *Hin*dIII-*Xba*I site of pAQN-EHX (designated pEBSTC).

(3) Modification of pBSTN and construction of expression plasmid: A 1.6-kbp *Hin*dIII fragment derived from pBSTN was ligated into the *Hin*dIII site of the multiple cloning sites of M13mp19. The plasmid DNA with the DNA region encoding the NH_2-terminal amino acid sequence of sialyltransferase 0160 on the *Eco*RI site side in the multiple cloning sites of M13mp19 was selected, and named pMBSTN. To introduce an *Eco*RI site just upstream of methionine, site-directed mutagenesis was carried out using primer MTY03, and the resulting plasmid was designated pMBSTN-E. The *Eco*RI-*Hin*dIII fragment of pMBSTN-E was ligated into the *Eco*RI-*Hin*dIII site of pEBSTC. The resulting plasmid was designated pEBST (Fig. 5). The *bst* gene could be inserted only into the plasmid containing the *lacI*^{q} 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 I).

COOH-Terminal Membrane-Binding Site—To introduce a stop codon at 539 L and 498 D, site-directed mutagenesis were performed on pMBSTC⊿X using primers MTY01 and MTY02, respectively. The resulting plasmids were named pMBSTC⊿137 and pMBSTC⊿178, respectively. These plasmid DNAs were digested with *Hin*dIII and *Xba*I. The *Hin*dIII-*Xba*I fragments were ligated into the *Hin*dIII-*Xba*I site of pAQN-EHX. The resulting plasmids were designated pEBSTC⊿137 and pEBSTC⊿178, respectively. Using these plasmids, two expression plasmids were constructed by the same methods as described in "*Construction of Expression Plasmid*," and named pEBST⊿137 and pEBST⊿178, respectively.

From the cells of *E. coli* MV1184 carrying pEBST⊿137 and pEBST⊿178, soluble-form enzyme was obtained



Expression plasmid, pEBST series

(B)

CACAGGAAACAGAATTCATGAAGAAA

S.D. *EcoRI* M K K

Fig. 5. **Structure of expression plasmids, pEBST series, for the sialyltransferase 0160 gene.** (A) Expression plasmids, pEBST series, were constructed from plasmid pAQN (*32*), pBSTN and pBSTC. (B) The nucleotide sequence around the initiation site of the pEBST series. The S.D. sequence derived from pAQN is indicated by boxes. The introduced *Eco*RI site is underlined.

TABLE I. **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	25.2 U/liter	N.D.
pEBST⊿137	56.6 U/liter	37.3 U/liter
pEBST⊿178	224.5 U/liter	121.3 U/liter

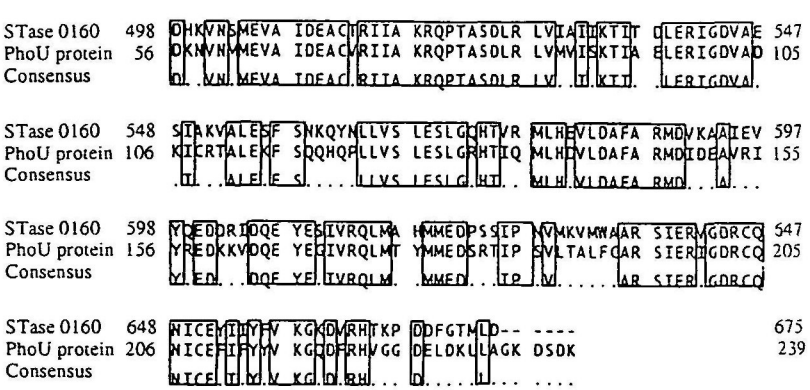


Fig. 4. **Comparison of amino acid sequences of sialyltransferase 0160 and PhoU protein.** The amino acid sequences numbered from the NH_2-terminal residue of the proteins. STase: The amino acid sequence of sialyltransferase 0160 [amino acid residue, 498 (D) to 675 (D)]. PhoU protein: The amino acid sequence of the phosphate transport system regulatory protein [amino acid residue, 56 (D) to 239 (K)]. Consensus amino acids are shown at the bottom of each line.

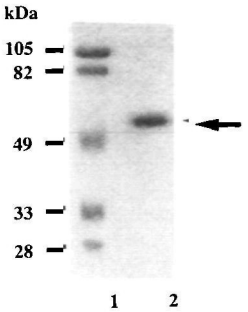


Fig. 6. **SDS-PAGE of purified recombinant sialyltransferase.** The electrophoresis was carried out on a 10% polyacrylamide gel at pH 8.4 in Tris-glycine buffer. Proteins were stained with Coomassie Brilliant Blue R-250. Phosphorylase B (105 kDa), bovine serum albumin (82 kDa), ovalbumin (49 kDa), carbonic anhydrase (33.3 kDa), and soybean trypsin inhibitor (28.6 kDa) were used as standards for molecular mass determination. Lane 1, standard proteins; molecular masses are indicated. Lane 2, recombinant sialyltransferase.

(Table I).

Purification of Recombinant Sialyltransferase 0160— Recombinant sialyltransferase 0160 was purified from the cells of *E. coli* MV1184 carrying pEBST⊿178 *via* the procedures described in "MATERIALS AND METHODS." The purified recombinant enzyme migrated as a single polypeptide with a molecular mass of 54 kDa under denaturing conditions (Fig. 6). The specific activity of purified recombinant sialyltransferase 0160 was 7.0 U/mg.

Using pyridylaminated lactose as an acceptor substrate, the enzymatic reaction was carried out and reaction products were analyzed by HPLC. The retention time of pyridylaminated lactose was 5.2 min. The retention time of the reaction product using sialyltransferase 0160 purified from *P. damsela* JT0160 was 6.0 min and that of the product using recombinant sialyltransferase 0160 was also 6.0 min. These results indicated that recombinant sialyltransferase 0160 transfers NeuAc from CMP-NeuAc to the galactose residue of lactose at position 6.

DISCUSSION

In order to clarify the primary structure of sialyltransferase 0160, we have cloned, sequenced and expressed in *E. coli* the gene coding for sialyltransferase 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-sialyltransferase using polyclonal antibodies raised against the purified enzyme (*33*). Up to present, 12 sialyltransferase 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 sialyltransferase, CMP-NeuAc (*16*). On the other hand, the bacterial α2,3-sialyltransferase genes from *Neisseria meningitidis* and *Neisseria gonorrhoeae* were cloned and expressed in *E. coli*. The genes encoding bacterial α2,3-sialyltransferases showed no similarity to those of mammalian sialyltransferases (*35*). The results of homology search showed that our sialyltransferase 0160 has no homologous regions to the cloned sialyltransferases, even though sialyltransferase 0160 shares some features of donor substrate and acceptor substrate specificity. These results indicated that sialyltransferase 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 sialyltransferase 0160 seems to be quite different from those of known sialyltransferases. The deduced primary sequences of the cloned sialyltransferases predicted a short NH_2-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, sialyltransferase 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 pEBST⊿137 and pEBST⊿178, Table I). From these results, it was predicted that (1) the catalytic domain of sialyltransferase 0160 may lie in the NH_2-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 sialyltransferase 0160 from *P. damsela* JT0160 has an NH_2-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 NH_2- 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 sialyltransferase 0160 is in progress.

P. damsela JT0160 is Gram-negative bacterium, and there is a candidate signal peptide in the NH_2-terminal region of the deduced amino acid sequence of sialyltransferase 0160. Thus, sialyltransferase 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 sialyltransferase 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 *Hin*dIII 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 that containing the *lacI*^{q} gene as the host plasmid.

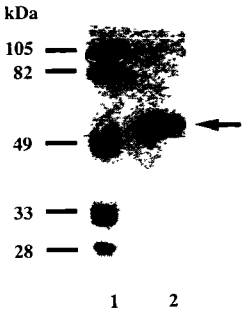


Fig. 6. **SDS-PAGE of purified recombinant sialyltransferase.** The electrophoresis was carried out on a 10% polyacrylamide gel at pH 8.4 in Tris-glycine buffer. Proteins were stained with Coomassie Brilliant Blue R-250. Phosphorylase B (105 kDa), bovine serum albumin (82 kDa), ovalbumin (49 kDa), carbonic anhydrase (33.3 kDa), and soybean trypsin inhibitor (28.6 kDa) were used as standards for molecular mass determination. Lane 1, standard proteins; molecular masses are indicated. Lane 2, recombinant sialyltransferase.

(Table I).

Purification of Recombinant Sialyltransferase 0160— Recombinant sialyltransferase 0160 was purified from the cells of *E. coli* MV1184 carrying pEBST⊿178 *via* the procedures described in "MATERIALS AND METHODS." The purified recombinant enzyme migrated as a single polypeptide with a molecular mass of 54 kDa under denaturing conditions (Fig. 6). The specific activity of purified recombinant sialyltransferase 0160 was 7.0 U/mg.

Using pyridylaminated lactose as an acceptor substrate, the enzymatic reaction was carried out and reaction products were analyzed by HPLC. The retention time of pyridylaminated lactose was 5.2 min. The retention time of the reaction product using sialyltransferase 0160 purified from *P. damsela* JT0160 was 6.0 min and that of the product using recombinant sialyltransferase 0160 was also 6.0 min. These results indicated that recombinant sialyltransferase 0160 transfers NeuAc from CMP-NeuAc to the galactose residue of lactose at position 6.

DISCUSSION

In order to clarify the primary structure of sialyltransferase 0160, we have cloned, sequenced and expressed in *E. coli* the gene coding for sialyltransferase 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-sialyltransferase using polyclonal antibodies raised against the purified enzyme (*33*). Up to present, 12 sialyltransferase 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 sialyltransferase, CMP-NeuAc (*16*). On the other hand, the bacterial α2,3-sialyltransferase genes from *Neisseria meningitidis* and *Neisseria gonorrhoeae* were cloned and expressed in *E. coli*. The genes encoding bacterial α2,3-sialyltransferases showed no similarity to those of mammalian sialyltransferases (*35*). The results of homology search showed that our sialyltransferase 0160 has no homologous regions to the cloned sialyltransferases, even though sialyltransferase 0160 shares some features of donor substrate and acceptor substrate specificity. These results indicated that sialyltransferase 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 sialyltransferase 0160 seems to be quite different from those of known sialyltransferases. The deduced primary sequences of the cloned sialyltransferases predicted a short NH_2-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, sialyltransferase 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 pEBST⊿137 and pEBST⊿178, Table I). From these results, it was predicted that (1) the catalytic domain of sialyltransferase 0160 may lie in the NH_2-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 sialyltransferase 0160 from *P. damsela* JT0160 has an NH_2-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 NH_2- 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 sialyltransferase 0160 is in progress.

P. damsela JT0160 is Gram-negative bacterium, and there is a candidate signal peptide in the NH_2-terminal region of the deduced amino acid sequence of sialyltransferase 0160. Thus, sialyltransferase 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 sialyltransferase 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 *Hin*dIII 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 that containing the *lacI*^{q} gene as the host plasmid.

This indicates that *bst* 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 (pEBSTΔ137) and 120 units/liter (pEBSTΔ178) of culture (Table I). On the other hand, mammalian Galβ1, 4GlcNAc α2,6-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 α2,6-sialyltransferase.

It is now possible to produce soluble-form α2,6-sialyltransferase on a large scale. This should be valuable for large-scale enzymatic synthesis of α2,6-sialylated oligosaccharides.

REFERENCES

1. Paulson, J.C. (1989) Glycoproteins: what are the sugar chains for? *Trends Biochem. Sci.* **14**, 272-276
2. Hakomori, S. (1990) Bifunctional role of glycosphingolipids. *J. Biol. Chem.* **265**, 18713-18716
3. Lasky, L.A. (1992) Selectins: Interpreters of cell-specific carbohydrate information. *Science* **258**, 964-969
4. Feizi, T. (1985) Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. *Nature* **314**, 53-57
5. Schauer, R. (1982) Chemistry, metabolism, and biological functions of sialic acids in *Advances in Carbohydrate Chemistry and Biochemistry* (Tipson, R.S. and Horton, D., eds.) Vol. 40, pp. 131-234, Academic Press, New York
6. Paulson, J.C., Beranek, W.E., and Hill, R.L. (1977) Purification of a sialyltransferase from bovine colostrum by affinity chromatography on CDP-agarose. *J. Biol. Chem.* **252**, 2356-2362
7. Weinstein, J., de Souza-e-Silva, U., and Paulson, J.C. (1982) Purification of a Galβ1,4GlcNAc α2,6-sialyltransferase and Galβ1,3(4)GlcNAc α2,3-sialyltransferase to homogeneity from rat liver. *J. Biol. Chem.* **257**, 13835-13844
8. Joziasse, D.H., Bergh, M.L.E., ter Hart, H.G.J., Koppen, P.L., Hooghwinkel, G.J.M., and van den Eijnden, D.H. (1985) Purification and enzymatic characterization of CMP-sialic acid:β-galactosyl-1,3-*N*-acetylgalactosaminide α2,3-sialyltransferase from human placenta. *J. Biol. Chem.* **260**, 4941-4951
9. Miyagi, T. and Tsuiki, S. (1982) Purification and characterization of β-galactoside(α2,6)sialyltransferase from rat liver and hepatomas. *Eur. J. Biochem.* **126**, 253-261
10. Wen, D.X., Livingstone, B.D., Medzihradsky, K.F., Kelm, S., Burlingame, A.L., and Paulson, J.C. (1992) Primary structure of Galβ1,3(4) GlcNAc α2,3-sialyltransferase: determined by mass spectrometry sequence analysis and molecular cloning. *J. Biol. Chem.* **267**, 21011-21019
11. Livingstone, B.D. and Paulson, J.C. (1993) Polymerase chain reaction cloning of a developmentally regulated member of the sialyltransferase gene family. *J. Biol. Chem.* **268**, 11504-11507
12. Kitagawa, H. and Paulson, J.C. (1994) Cloning of a novel α2,3-sialyltransferase that sialylates glycoproteins and glycolipids carbohydrate groups. *J. Biol. Chem.* **269**, 1394-1401
13. Kurosawa, N., Kojima, N., Inoue, M., Hamamoto, T., and Tsuji, S. (1994) Cloning and expression of Galβ1,3GalNAc-specific GalNAc α2,6-sialyltransferase. *J. Biol. Chem.* **269**, 19048-19053
14. Kurosawa, N., Hamamoto, T., Lee, Y.C., Nakaoka, T., Kojima, N., and Tsuji, S. (1994) Molecular cloning and expression of GalNAc α2,6-sialyltransferase. *J. Biol. Chem.* **269**, 1402-1409
15. Yoshida, Y., Kojima, N., and Tsuji, S. (1995) Molecular cloning and characterization of a third type of *N*-glycan α2,8-sialyltransferase. *J. Biochem.* **118**, 658-664
16. Datta, A.K. and Paulson, J.C. (1995) The sialyltransferase "sialyl motif" participates in binding the donor substrate CMP-NeuAc. *J. Biol. Chem.* **270**, 1497-1500
17. Yamamoto, T., Nakashizuka, M., Kodama, H., Kajihara, Y., and Terada, I. (1996) Purification and characterization of a marine bacterial β-galactoside-α2,6 sialyltransferase from *Photobacterium damsela* JT0160. *J. Biochem.* **120**, 104-110
18. Kajihara, Y., Yamamoto, T., Nagae, H., Nakashizuka, M., Sakakibara, T., and Terada, I. (1996) A novel α-2,6-sialyltransferase: Transfer of sialic acid to fucosyl and sialyl trisaccharides. *J. Org. Chem.* **61**, 8632-8635
19. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Saito, H. and Miura, K. (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta* **72**, 619-629
21. Hanahan, D. and Meselson, M. (1980) Plasmid screening at high colony density. *Gene* **10**, 63-67
22. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
23. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**, 10035-10038
24. Kondo, A., Suzuki, J., Kuraya, N., Hase, S., Kato, I., and Ikenaka, T. (1990) Improved method for fluorescence labeling of sugar chains with sialic acid residues. *Agric. Biol. Chem.* **54**, 2169-2170
25. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488-492
26. Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* **157**, 105-132
27. Amemura, M., Makino, K., Shinagawa, H., Kobayashi, A., and Nakata, A. (1985) Nucleotide sequence of the genes involved in phosphate transport and regulation of the phosphate regulon in *Escherichia coli*. *J. Mol. Biol.* **184**, 241-250
28. Shine, J. and Dalgarno, L. (1975) Determination of cistron specificity in bacterial ribosomes. *Nature* **254**, 34-38
29. Harley, C.B. and Reynolds, R.P. (1987) Analysis of *E. coli* promoter sequences. *Nucleic Acid Res.* **15**, 595-623
30. Hawley, D.K. and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter sequences. *Nucleic Acids Res.* **11**, 2237-2255
31. Tinoco, I., Borer, P., Dengler, B., and Levine, M.D. (1973) Improved estimation of secondary structure in ribonucleic acids. *Nature* **246**, 40-41
32. Lee, Y.C., Miyata, Y., Terada, I., Ohta, T., and Matsuzawa, H. (1991) Involvement of NH_2-terminal pro-sequence in the production of active Aquqlysin I (a thermophilic serine protease) in *Escherichia coli*. *Agric. Biol. Chem.* **55**, 3027-3032
33. Weinstein, J., Lee, E.U., McEntee, K., Lai, P.H., and Paulson, J.C. (1987) Primary structure of β-galactoside α2,6-sialyltransferase. *J. Biol. Chem.* **262**, 17735-17743
34. Sasaki, K., Kurata, K., Kojima, N., Kurosawa, N., Ohta, S., Hanai, N., Tsuji, S., and Nishi, T. (1994) Expression cloning of a G_{M3}-specific α-2,8-sialyltransferase (G_{D3} synthase). *J. Biol. Chem.* **269**, 15950-15956
35. Gilbert, M., Watson, D.C., Cunningham, A.-M., Jennings, M.P., Young, N.M., and Wakarchuk, W.W. (1996) Cloning of the lipooligosaccharide α-2,3-sialyltransferase from the bacterial pathogens *Neisseia meningitidis* and *Neisseria gonorrhoeae*. *J. Biol. Chem.* **271**, 28271-28276
36. Recny, M.A., Grabau, C., Cronan, J.E., Jr., and Hager, L.P. (1985) Characterization of the α-peptide released upon protease activation of pyruvate oxidase. *J. Biol. Chem.* **260**, 14287-14291
37. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
38. Hamamoto, T., Lee, Y.C., Kurosawa, N., Nakaoka, T., Kojima, N., and Tsuji, S. (1994) Expression of mouse Galβ1,4GlcNAc-α2,6-sialyltransferase in an insoluble form in *Escherichia coli* and partial renaturation. *Bioorg. Med. Chem.* **2**, 79-84
39. Oliver, D. (1985) Protein secretion in *Escherichia coli*. *Annu. Rev. Microbiol.* **39**, 615-648