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Expression of α2,8/2,9-Polysialyltransferase from *Escherichia coli* K92

CHARACTERIZATION OF THE ENZYME AND ITS REACTION PRODUCTS*

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Gwo-Jenn Shen‡, Arun K. Datta‡, Masayuki Izumi, Kathryn M. Koeller, and Chi-Huey Wong§

From the Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037

The capsular polysaccharide of *Escherichia coli* K92 contains alternating -8-NeuAcα2- and -9-NeuAcα2- linkages. The enzyme catalyzing this polymerizing reaction has been cloned from the genomic DNA of *E. coli* K92. The 1.2-kilobase polymerase chain reaction fragment was subcloned in pRSET vector and the protein was expressed in the BL21(DE3) strain of *E. coli* with a hexameric histidine at its N-terminal end. The enzyme was isolated in the supernatant after lysis of the cells and fractionated by ultracentrifugation. Western blotting using anti-histidine antibody showed the presence of a band that migrated at about 47.5 kDa on both reducing and nonreducing SDS-polyacrylamide gel electrophoresis, indicating a monomeric enzyme. Among the carbohydrate acceptors tested, *N*-acetylneuraminic acid and the gangliosides G_{D3} and G_{Q1b} were preferred substrates. The cell-free enzyme reaction products obtained were characterized by NMR and mass spectrometry, which indicated the presence of both α2,9- and α2,8-linked polysialyl structure. The K92 *neuS* gene was used to transform the K1 strain of *E. coli*, the capsule of which contains only -8-NeuAcα2- linkages. Analysis of the polysaccharides isolated from these transformed cells is consistent with the presence of both -8-NeuAcα2- and -9-NeuAcα2- linkages. Our results suggest that the *neuS* gene product of *E. coli* K92 catalyzes the synthesis of polysialic acid with α2,9- and α2,8-linkages *in vitro* and *in vivo*.

The 9-carbon 5-amino-3,5-dideoxy-D-glycero-D-galactononulosonic acids or sialic acids, comprise a family of neuraminic acid derivatives which are widely spread in nature, ranging from bacterial to human origin. This sugar moiety linked to carbohydrate chains of glycolipids and glycoproteins plays a key role in many important biological events mediated by carbohydrate-protein interactions (1). Sialic acids are also found in linear homopolymers (polysialic acid) of *N*-acetylneuraminic acid (NeuAc)[1] and *N*-glycolylneuraminic acid (Neu5Gc) joined by α2,8-, α2,9-, or α2,8/α2,9-ketosidic linkages (2). The degree of polymerization can extend beyond 200 sialic acid residues (3), thereby constituting a structurally unique group of carbohydrate polymers which covalently modify cell-surface glycoconjugates (4). Polysialylation of the mammalian neural cell adhesion molecules, for example, affects the cell-cell adhesive interactions during embryogenesis, including neurite fasciculation and neuromuscular interactions (5, 6). While the neuroinvasive bacteria *Escherichia coli* K1 contain a homopolymer of -8-NeuAcα2- (7), the *Neisseria meningitidis* Group C (8, 9) displays a homopolymer of -9-NeuAcα2- linkages. On the other hand, the polysialic acid capsular antigens of neuroinvasive *E. coli* K92 are composed of poly(-8NeuAcα2,9NeuAcα2-) residues (10). Alternating linkages of α2,8NeuAc- and α2,9NeuAc- could not be found in mammalian systems, and therefore could be utilized to elicit immunological response. In fact, the heteropolymer from K92 strain of *E. coli* is being imployed as a carbohydrate vaccine in the clinical trial of patients suffering from acute meningitidis (11).

The sialyltransferases that catalyze the addition of sialic acid to form such diverse carbohydrate recognition molecules fall into at least two families. While 13 clones have been obtained from mammalian systems (12), only 4 have been cloned from bacterial systems to date (13–16). Recent cloning of both mammalian and bacterial sialyltransferases showed that these two gene families evolved differently. The polysialyltransferase encoded by *neuS* in *E. coli* uses CMP-sialic acid as a donor and catalyzes a sequential addition of sialic acid to the nonreducing end of an acceptor (3, 17, 18). In a detailed study using the pathogenic strain of *E. coli* K1, the gene coding for CMP-Neu5Ac:poly-α2,8-sialosyl sialyltransferase, termed *neuS*, has been cloned. This gene was found to be clustered in the *kps* region which is implicated in the initiation, polymerization, and possibly translocation of polysialic acid chains across the inner membrane (13, 19, 20). A similar gene has been identified by hybridization to the K92 strain of *E. coli*, the capsule of which contains poly(-8NeuAcα2,9NeuAcα2-) (10). The complete nucleotide sequence of this K92 *neuS* gene showed that this gene product differs by 70 amino acids from the K1 *neuS* gene product (14). Although an *in vitro* study indicated that the *neuS* from *E. coli* K1 coding for a 47-kDa protein has polysialyltransferase activity, no such activity has yet been clearly

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‡ Contributed equally to this work.

§ To whom correspondence should be addressed: Dept. of Chemistry, BCC 338, The Scripps Research Institute, 10550 N. Torrey Pines Rd., San Diego, CA 92037. Tel: 858-784-2487; Fax: 858-784-2409; E-mail: wong@scripps.edu.

[1] The abbreviations used are: NeuAc, neuraminic acid; PCR, polymerase chain reaction; CMP-NeuAc, CMP-neuraminic acid or CMP-sialic acid; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; ES-MS, electrospray mass spectrometry; G_{M3}, NeuAcα2,3Galβ1,4Glcβ-ceramide; G_{D3}, NeuAcα2,8NeuAcα2,3Galβ1,4Glcβ-ceramide; G_{Q1b}, NueAcα2,8 NeuAcα2,3Galβ1,3GalNAcβ1,4(NueAcα2,8Neuα2,3)Galβ1,4Glcβ-ceramide; G_{D2}, GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4Glcβ-ceramide; G_{T1a}, NueAcα2,3Galβ1,3(NeuAcα2,6)GalNAcβ1,4(Neu Acα2,3)Galβ1,4Glcα-ceramide; G_{T1b}, NeuAcα2,3Galβ1,3GalNAcβ1,4 (NeuAcα2,8 NeuAcα2,3)Galβ1,4Glcβ-ceramide.

demonstrated for the *neuS* product from *E. coli* K92. In this study, we have cloned the *neuS* gene from the K92 strain of *E. coli* by PCR using the primers described previously (14), expressed the gene in *E. coli*, and shown that the enzyme from this gene catalyzes the synthesis of polysialic acids with both α2,8- and α2,9-linkages *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cloning of K92 neuS Gene—The genomic DNA isolated from *E. coli* strain K92 (ATCC 35860) using a DNA extraction kit (Qiagen Co., Valencia, CA) was used for the amplification of the *neuS* gene by PCR. This was performed in a 100-μl reaction mixture containing 100 ng of genomic DNA as template, 300 nmol of primers, 200 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and a mixture of both *Taq* DNA polymerase and *pfu* DNA polymerase (1 unit of each). Two primers, the forward primer with internal *Bam*HI (underlined) site (5′-ATATTGGATCC)ATATTTGATGCTAGTTTA-3′; nucleotides 3–20) and a reverse primer with EcoRI site, (5′-GGCGCGAATTCTTACTCCCCCAAGAAAA-3′; nucleotides 1230–1214) designed based on the previously published sequence (14) were used for PCR using the following conditions: 94 °C, 1 min; 50 °C, 1 min; and 72 °C, 2 min for 35 cycles. Agarose gel analysis of the product showed the presence of one major band with the expected size of about 1.2 kilobases. The fragment obtained was purified by agarose gel electrophoresis, digested with *Bam*HI and *Eco*RI, and subcloned into a similarly digested pRSET vector (Invitrogen Co., San Diego, CA) following standard molecular biological techniques (21). The ligation mixture was then used for transformation of competent cells of the BL21(DE3) strain of *E. coli* (Novagen, Madison, WI). The clone containing the expression vector was confirmed by sequencing of both strands using an automated DNA sequencer (ABI 377) at the TSRI Core facility.

Expression of the K92 neuS Gene—Since the DH5α strain of *E. coli* lacking the *kps* gene cluster does not have sialyltransferase activity (18), it was used initially to express the gene. This strain has a pilli forming gene in its chromosome and therefore does not require any pressure for the expression of its sex pilli. M13 phage containing the T7 RNA polymerase gene (Invitrogen) was used to infect DH5αF′ transformed with pRSET vector containing the cloned *neuS* gene. The transformed cells were grown in LB medium (with 1 mM $MgCl_2$) containing 100 μg of carbicillin/ml at 37 °C to A_{600} ~0.3 and then 1 mM IPTG was added. After 1 h of incubation, the M13/T7 phage was added to infect the cells as described by the supplier (Invitrogen), and harvested after 5 h of incubation. An enzyme activity assay and SDS-PAGE of the cell lysate (15 K supernatant) showed expression of the *neuS* gene in DH5αF′. A band migrating as ~47.5 kDa in the 12% SDS-PAGE appeared within 1 h of incubation after infection with the T7 RNA polymerase gene containing M13 in the presence of IPTG (as judged by Western blot). The relative intensity of the 47.5-kDa band increased with time and reached maximum after 4 h. Enzymatic assay of the cell-free extract using sialic acid as acceptor also indicated the expression of the K92 polysialyltransferase. However, the DH5αF′ cells transformed with pRSET vector also yielded a band comigrating with the 47.5-kDa band in SDS-PAGE. Attempts to separate these bands using Ni^{2+}-NTA-agarose proved to be difficult. We therefore decided to express this protein in the HMS174(DE3)Lys or BL21(DE3) strain of *E. coli* (Invitrogen).

The transformed BL21(DE3) cells containing the *neuS* gene from the K92 strain of *E. coli* were grown exponentially in LB medium (with 1 mM $MgCl_2$) containing 100 μg of carbicillin/ml at 37 °C until A_{600} ~ 0.3 was reached. Expression of the *neuS* gene was achieved at 37 °C by induction with IPTG as described in the expression kit (Invitrogen, Carlsbad, CA). The cells were harvested after 5 h of incubation.

Western Blot—This analysis was done by following a standard technique (22). The protein samples were boiled for 8–10 min in 1× Laemmli gel sample buffer either in the absence or presence of 10% β-mercaptoethanol. Electrophoresis was performed using 10% SDS-polyacrylamide gel in Tris/glycine SDS buffer. After the proteins were transferred to a nitrocellulose membrane, the blot was blocked with a blocking buffer (blotto in TBS and 0.1% Tween 20) and developed by adsorption with the mouse anti-$(His)_5$ monoclonal antibody (Qiagen, Valencia, CA) diluted (1:500) with the blocking buffer. The blot was incubated with horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (1:1000; Amersham Pharmacia Biotech). The protein bands were visualized either by chemiluminescence as suggested by the supplier of the reagents (Amersham Pharmacia Biotech) or by staining with the immunoPure Metal Enhanced DAB Substrate Kit (Pierce, Rockford, IL).

Sialyltransferase Assay—The assay was carried out essentially as described by Vimr and co-workers (23) with the following modifications: after harvesting, the cells were washed three times with 25 mM Tris-HCl (pH 8.0) and then resuspended in 25 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), and 1 μl/ml protease inhibitor mixture (Sigma). Cells were lysed three times by using a French Press (Aminco; 1,500 p.s.i.). The cell debris was removed by centrifugation twice at 15,000 rpm and the supernatant was collected (termed the 15 K Sup). This supernatant was then centrifuged again at 100,000 × *g* (~40,000 rpm) using a Beckman ultracentrifuge (Model L5–50). Both the supernatant (termed the 40 K Sup) and the pellet were saved. The pellet was resuspended in a buffer containing 25 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM DTT, and 0.1% Triton CF-54. The supernatants and the pellet were stored on ice, and used for assay within 2 h.

Without the addition of acceptor, the endogenous enzyme activity was determined. The exogenous activity was then detected by addition of sialic acid as acceptor and CMP-[^{14}C]NeuAc as donor in a reaction buffer containing 25 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM DTT, and 0.5% Triton CF-54. Other substrates were also used, including colominic acid. Both the endogenous and exogenous enzyme activity assays measure the transfer of ^{14}C-labeled NeuAc to the acceptor. Unreacted radiolabeled substrates were separated by DEAE-cellulose TLC (1-propanol, 14 M NH_4OH/H_2O, 6:1:3) or by Sephadex G-25 column chromatography. Radioactivity remaining at the origin and/or at the top of the TLC plate or the product obtained at the void volume of Sephadex G-25 column, was quantitated by liquid scintillation spectrometry. For analysis using Sephadex G-25 column chromatography, a column (1 × 3.5 cm) of Sephadex G-25 (Amersham Pharmacia Biotech) in water was prepared. The radiolabeled cell-free reaction mixture (60 μl) was loaded and washed with 1.5 ml of water, which was collected and counted. In some experiments, the activity was determined by separating the unused CMP-[^{14}C]NeuAc by Dowex 1X8 (phosphate form) column chromatography (24). The initial rates were estimated graphically, and activity was expressed as picomoles of sialic acid transferred per hour.

Thin-layer Chromatography and Fluorography—After the cell-free reaction was complete, the proteins were removed by an Amicon filter (MWCO 10 K), and the supernatant containing oligomeric reaction products was separated by Silica Gel 60 (aluminum backed) and/or DEAE-cellulose (plastic backed) thin-layer chromatography (TLC) using the following solvent systems: (a) 1-propanol, 14 M NH_4OH, H_2O, 6:1:3; (b) chloroform, methanol, 0.02% $CaCl_2$, 30:55:15. Control chromatograms were calibrated with mono- to hexasialosyl oligomers (Calbiochem, San Diego, CA) which were detected by orcinol spray, as described by Corfield and Schauer (25). This method resolved monomeric through octameric sialic acids. Oligomers composed of nine or more sialosyl residues were retained at the origin. Labeled chromatograms were sprayed with En^{3}Hance (NEN Life Science Products Inc.) and fluorographed against an intensifier screen. After fluorography, sialosyl oligomers were detected on Silica Gel 60 TLC plates by orcinol spray for alignment of radioactive signals with colorimetrically detected bands. For analysis using DEAE-cellulose TLC, the position of the cell-free ^{14}C-labeled reaction product(s) was marked after alignment of radioactive signals, cut and counted.

Isolation and Purification of Polysialylated Oligosaccharides—Strains of *E. coli* transformed with vector only or with the K92 *neuS* gene were used for the extraction of polysaccharide following the method of Kasper *et al.* (26) with the following modifications. For K92 polysaccharide, the K92 strain of *E. coli* was grown in 2 liters of Davis' supplemental minimal medium (ATCC media formulation number 54) at 37 °C overnight. The K1 strain of *E. coli* transformed with the K92 *neuS* gene was grown in 2 liters of Davis' supplemental minimal medium containing 2% glycerol instead of glucose. The cells were grown to A_{600} ~ 0.3 and 1 mM IPTG (final concentration) was added. After incubation at 37 °C for 1 h, M13/T7 phages (Invitrogen; 10 plaque forming units/cell) were added and incubated at 37 °C. After overnight growth the organisms were removed by centrifugation (48,000 × *g* for 30 min at 4 °C), and the supernatant was collected and filtered through a 0.45-μm Millipore filter. Hexadecyltrimethyl ammonium bromide (Cetavlon, 0.3%; Sigma) was added to the supernatant with stirring and the precipitate formed was collected by centrifugation. The precipitate was solubilized in 0.9 M $CaCl_2$ and reprecipitated with 3 volumes of chilled (−20 °C) absolute ethanol. The ethanol precipitation from 0.9 M $CaCl_2$ was repeated three times. The final product was dialyzed against water and lyophilized. The final purification was done using a column (2 × 25 cm) of Bio-Gel P-2 (Amersham Pharmacia Biotech) eluted with water following the usual procedure, and verified by polyacrylamide gel electrophoresis following the method of Troy and McCloskey (7). After hydrolysis by sulfuric acid (0.1 N H_2SO_4, 80 °C), quantitation of sialic

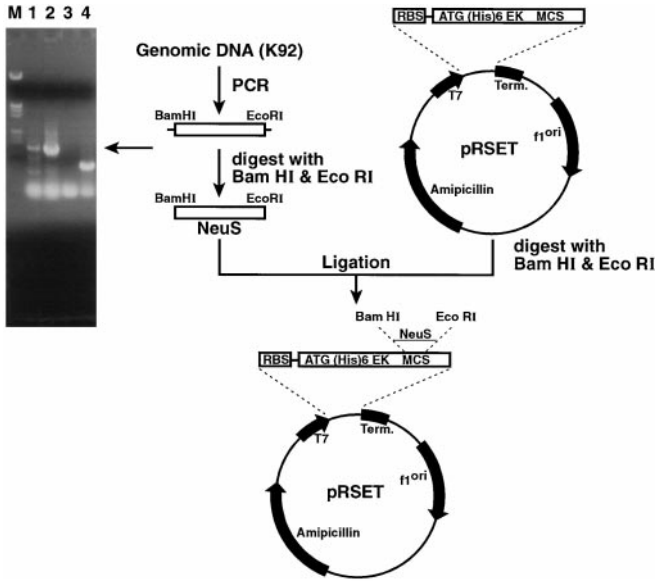

FIG. 1. **Construction of the expression vector for the K92 neuS expression as a fusion protein.** The PCR fragment, 91.2 kilobases, obtained for the full-length form of the K92 *neuS* gene using its genomic DNA as template, was subcloned in pRSET vector (Novagen, Madison, WI) using two unique restriction sites, *Bam*HI and *Eco*RI. This resulted in the expression of the protein with a hexameric histidine tag fused at its N-terminal end. The vector provides the start codon followed by the sequence for the hexameric histidine. The full-length form of the protein remains mostly as membrane bound form. However, about 30–40% of the protein is "released" in the 40 K Sup upon treatment with a detergent. This released protein retains full catalytic activity and was used in this study.

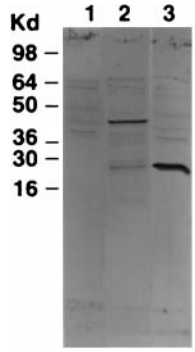

FIG. 2. **Western blot analysis of the K92 neuS expressed protein.** *E. coli* cells of BL21(DE3) transformed with K92 *neuS* gene in the expression vector pRSET were induced with IPTG (1 mM final concentration) and harvested after 4 h. Cells transformed with pRSET vector only were also grown and used as negative control. As a positive control, BL21(DE3) previously transformed with the histidine-tagged thioesterase gene subcloned in pET20B(+) were grown, induced, and harvested similarly. After harvesting, the cells were lysed and the supernatants were collected after centrifugation at 15,000 rpm (15 K Sup). The proteins were run using a 12% SDS-PAGE as mentioned under "Materials and Methods." The samples were: 15 K Sup from the negative control (*lane 1*), 15 K Sup from the cells transformed with K92 *neuS* gene (*lane 2*), and 15 K Sup from the positive control (*lane 3*). The blot was developed using anti-$(His)_5$ antibody (Qiagen) and metal enhanced DAB substrate kit (Pierce). The neuS expressed α2,8/2,9-polysialyltransferase migrated in the gel at about 47.5 kDa. In *lane 3*, the positive control for the Western blot showed a band at ~23 kDa for the thioesterase. It may be noted that the expression level for the *neuS* gene was comparatively lower than the thioesterase gene.

acid was determined by the thiobarbituric acid procedure as described previously (27).

Protein Assay—This was done using bicinconic acid (BCA) reagent following the instructions provided by the supplier (Pierce).

Mass Spectrometry Analysis—The electrospray ionization (ES-MS) mass spectrometry experiments were performed on an API III Perkin-Elmer SCIEX triple quadrupole mass spectrometer. Electrospray samples were typically introduced to the mass analyzer at a rate of 4.0 ml/min. The positive and negative ions, generated by charged droplet evaporation, enter the analyzer through an interface plate and a 100-mm orifice, while the declustering potential is maintained between 50 and 200 V to control the collisional energy of the ions entering the mass analyzer. The emitter voltage is typically maintained at 5000 V. The m/z was determined for the major peaks.

NMR Analysis—^{13}C nuclear magnetic resonance spectra were recorded at about 23 °C on a Bruker AMX-400 or DRX-600 spectrometer operating in the pulsed Fourier transform mode using broad band proton noise decoupling. The polysaccharides were run as deuterium oxide solutions at pH 7.0. The solvent, D_2O, served as an internal lock signal. dimethyl sulfoxide was often used as internal reference. The ^{13}C spectrum of polysaccharides from K1 and K92 determined in this study were found to be identical to that presented by Egan *et al.* (10). The K1 polysaccharide NH*C*O (amide carbonyl) resonances from the present and Egan's studies were taken to have the same chemical shift and all remaining signals referenced accordingly.

RESULTS

Cloning of the neuS Gene from K92—Previously, the *neuS* gene from the K1 strain of *E. coli* was cloned and found to have the α2,8-polysialyltransferase activity. Using the K1 *neuS* gene as a probe, Vimr *et al.* (14) cloned a 1230-base pair gene (14) from the K92 strain of *E. coli* that had 87.3% homology to the K1 gene. The deduced protein sequence from this gene indicated the same number of amino acids (total 409 amino acids) with 83% homology to that of K1 at the protein level. Previous structural studies on the polysaccharide antigen of this *E. coli* K92 strain (Bos-12) suggested the presence of polysialic acid with alternating α2,8- and α2,9-linkages (10). Therefore, the gene product from the K92 strain is assumed to confer polysialyltransferase activity (14, 23). However, expression of the *neuS* gene from K92 was not clearly demonstrated, nor was it shown to form a polymer of sialic acid *in vitro* with alternating α2,8/2,9-linkages.

To characterize the K92 *neuS* gene product, we have cloned this gene from the *E. coli* K92 strain using a PCR based approach. The primers were designed based on the previously published sequence (14), and used to amplify the full-length clone of the *neuS* gene from the genomic DNA of the K92 strain. The 1.23-kilobase fragment obtained by PCR was subcloned in pRSET (Invitrogen) for expression of the *neuS* gene product as a fusion protein with the hexameric histidine tag fused at its N-terminal end (Fig. 1). The clone was verified by double-strand sequencing of the entire fragment including the restriction sites.

A hydrophilicity plot (28, 29) revealed two potential membrane-spanning regions in this protein of 409 amino acid residues. The first one is located 177 residues from the N terminus. It consists of 19 hydrophobic amino acids bordered by three upstream lysine residues. Interestingly, this first transmembrane domain is bordered on either end by a proline residue. Proline is known to confer conformational constraints of many biologically important proteins, as evidenced by site-directed mutagenesis studies (see Ref. 30, for a review, see Ref. 31). A similar KKKP motif, found to be present in the ST6Gal I (for nomenclature, see Ref. 12) polypeptide, is part of a proposed signal anchor sequence (32). The second transmembrane domain is located 327 residues from the N terminus. It consists of 17 hydrophobic amino acid residues bordered by two upstream lysine residues and a proline residue at its C terminus. Notably, lysine and serine are predominantly present in this domain. The presence of these two transmembrane domains suggests that this protein is membrane-bound as further supported by the Western blot (see below). This feature appears to be unique among the cloned bacterial sialyltransferases, the hydrophilicity plot of which suggests the presence of only one potential membrane-spanning region.

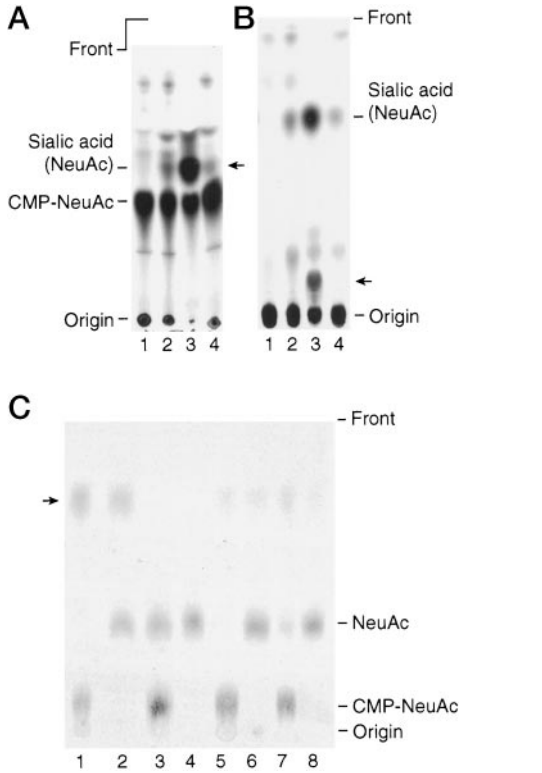

FIG. 3. **Analysis of the enzyme reaction products by thin-layer chromatography (TLC).** The 40 K Sup was used as a source of the enzyme in the reaction mixture containing 1 mM acceptor and CMP-[^{14}C]NeuAc (9 nmol; 1 μCi) as donor in a reaction buffer containing 25 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM DTT, and 0.5% Triton CF-54. As an example, analysis was shown with the reaction mixture containing sialic acid and G_{M3} as acceptors and CMP-[^{14}C]NeuAc as donor substrate. As a negative control, the reaction mixture contained sialic acid as acceptor without the addition of any enzyme. For *A* and *B*, Silica Gel 60 TLC plates were used for the samples: reaction mixture without the enzyme (*lane 1*), G_{M3} as acceptor (*lane 2*), sialic acid as acceptor (*lane 3*), and reaction mixture without exogenous sialic acid (*lane 4*). The solvents used were: (a) 1-propanol, 1 M NH_4OH, water; 7:1:2; (b) chloroform, methanol, 0.02% $CaCl_2$; 45:55:10. The reaction mixtures were also analyzed by DEAE-cellulose TLC. After the reaction was complete, a part of the sample was acidified with 10% acetic acid and incubated for 30 min at room temperature. For *C*, DEAE-cellulose TLC plate was used. The samples were: reaction mixture with sialic acid as acceptor (*lane 1*) and after acidification (*lane 2*); reaction mixture without enzyme (*lane 3*) and after acidification (*lane 4*); using G_{M3} as acceptor (*lane 5*) and after acidification (*lane 6*); reaction mixture without exogenous acceptor (*lane 7*) and after acidification (*lane 8*). The solvents in *C* used were: 1-propanol, 14.5 M NH_4OH, water; 6:1:3. The plates were dried after the run and exposed to Kodak XAR-5 film. The *arrow* indicates the position of the potential cell-free reaction product(s). The position of CMP-NeuAc and free sialic acid were also shown. We found that both the enzyme and its reaction products are unstable and degrade over time.

Expression of the K92 neuS Gene—For expression, we used pRSET (Invitrogen), a prokaryotic expression vector designed to obtain a hexameric histidine tag fused at the N-terminal sequence of the desired protein. Due to the lack of any antibody available against the K92 *neuS* gene product, the polysialyltransferase was expressed as a fusion protein, and detected by the Western blot using anti-$(His)_5$ antibody (Qiagen Inc., Valencia, CA).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot—The cell-free crude extract (15 K Sup and 40 K Sup) and the cell pellet, suspended in 25 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$ and 0.1% Triton CF-54, obtained by ultracentrifugation were analyzed by SDS-PAGE. Coomassie Blue staining showed the presence of various bands including a major band at about 47.5 kDa, the expected size for the *neuS* gene product. This band was absent in the control (cells transformed with pRSET vector only). Replica gel was used to transfer the proteins to a nitrocellulose membrane and detected by using mouse monoclonal anti-$(His)_5$ antibody (Qiagen, Valencia, CA). The blot was finally developed by using Enhanced DAB substrates following the protocol of the supplier (Pierce) or by a chemiluminescence technique as described earlier (30). The Western blot clearly showed the presence of a single band at about 47.5 kDa (Fig. 2). The α2,8/2,9-polysialyltransferase was also found to be present in the 40,000 supernatant. Our initial attempt to purify the protein using Ni^{2+}-NTA-agarose column failed. Although the fusion protein apparently binds to the Ni^{2+}-NTA-agarose, nonspecific binding with other cellular proteins was also observed. Therefore, the 40 K Sup was used in this study for enzyme assay and analysis of its product(s).

TABLE I
Substrate specificity of the neuS gene product of E. coli K92 expressed in BL21(DE3)

The reaction mixture (60 μl) contained 1 μM each of the following acceptors and 9 nmol of CMP-[^{14}C]NeuAc (specific activity: 8000 cpm/nmol) as donor substrate. The reactions were carried out at 37 °C for 1 h using 20 μg of 40 K Sup as mentioned under "Materials and Methods." The reaction mixture (1 μl) was spotted on a plastic backed DEAE-cellulose TLC plate, and ran in 1-propanol, 14.5 M NH_4OH; water, 6:1:3. After air drying, the plate was exposed to Kodak XAR-5 film for overnight at −70 °C. The area corresponding to the reaction product was marked, cut and then counted in scintillation fluid.

Potential acceptor substrates tested	Counts/min obtained	Total sialic acid transferred
	per 60-μl reaction	*pmol*
Sialic acid (5-AcNeuAc)		
Monomer	3780	470
Dimer	1080	140
Trimer	1560	140
Pentamer	0	0
Hexamer	0	0
9-*O*-Acetylsialic acid	900	110
Colominic acid	0	0
G_{D3}	2880	360
G_{M3}	1440	180
G_{D2}	900	110
G_{T1a}	460	40
G_{T1b}	1140	130
G_{Q1b}	2460	310
Ganglioside mixtures	720	90

Sialyltransferase Assay—The cell-free crude extract (15 K Sup), 40 K supernatant (40 K Sup), and the pellet were used for the assay of various acceptor substrates and CMP-[^{14}C]NeuAc as donor substrate. The activity could be traced in all these fractions. After the reaction was over, the products were analyzed by TLC or Sephadex G-25 column chromatography. Fig. 3 shows a representative analysis. Using cold sialic acid as acceptor and CMP-[^{14}C]NeuAc as donor, a major spot was detected on TLC using various solvent systems. Interestingly, this new spot behaved as non-polar material on DEAE-cellulose TLC (Fig. 3*C*). It was found that sialic acid served as a good acceptor, whereas colominic acid did not (Table I). 9-*O*-Acetylsialic acid was not well accepted, indicating that polymerization probably starts at the C-9 position of the sialic acid acceptor. Notably, pentameric and hexameric polysialosides with α2,8-linkages were not accepted, whereas the corresponding dimer and trimer (Calbiochem) served as poor acceptors. Among the glycolipids tested, G_{D3} and G_{Q1b} also served as good acceptors. However, other sialylgangliosides tested were poor substrates (Table I).

Structural Determination of the Cell-free Reaction Product—For structural determination, the cell-free reactions were carried out, using the 40,000 supernatant as the enzyme source, with sialic acid and unlabeled CMP-NeuAc in a molar ratio of 1:10 to obtain the reaction product(s) in a sufficient quantity.

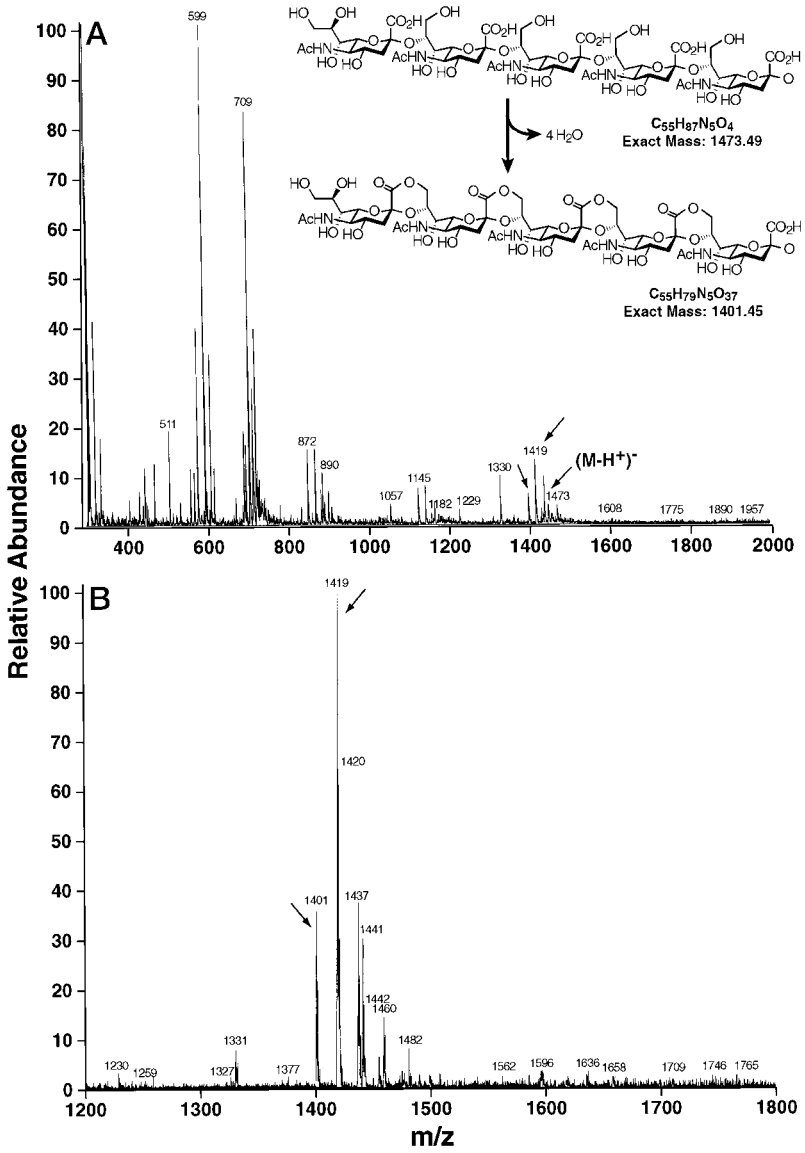

FIG. 4. **Mass-spectral analysis of the enzyme reaction product.** For the mass spectral analysis, the cell-free reaction was carried out using 40 K Sup. The reaction mixture contained 1 mM sialic acid and 10 mM CMP-NeuAc as donor in a reaction buffer containing 25 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM DTT, 0.5% Triton CF-54. To drive the forward reaction, the liberated CMP was removed by the addition of alkaline phosphatase (1 units). After the reaction was over, the reaction material was passed through an Amicon filter (10 MWCO) and the filtrate was used for analysis. The cell-free enzyme reaction product behaved similar to the pentameric sialic acid (Calbiochem), on TLC. in various solvent systems. Mass spectra were compared with that of commercially available pentameric sialic acid. Negative ion mass spectrum (ES-MS) of: *A,* standard pentameric polysialic acid; and *B,* cell-free reaction product. The major peaks for m/z were marked by an *arrow* and described in the text. The molecular ion $(M - H^+)^-$ was also shown. Some minor products with higher mass (m/z) were also observed.

Since it was known that the by-product CMP inhibits the forward reaction, alkaline phosphatase was added to remove CMP in order to increase the yield. As mentioned above, the product moved as a non-polar material on DEAE-cellulose TLC, a characteristic utilized for purification of the cell-free reaction product(s). After the reaction was complete, the reaction mixture was passed through a column of Dowex 1X8 (phosphate form) to remove unreacted CMP-NeuAc and sialic acid. The negative ion mass spectral analysis (ES-MS) showed (Fig. 4) a major peak at m/z 1419 for $(M\text{-}3H_2O{-}H^+)^-$ and at m/z 1401 for (M-4 H_2O − H). This spectrum was similar to that of the commercially obtained α2,8-polysialic acid (pentamer; Calbiochem). While the authentic sample showed a fragmentation pattern accounting for the tetramer (m/z at 1182 for $M\text{-}3H_2O{-}H^+)^-$, trimer (m/z at 890 for $M\text{-}3H_2O{-}H^+)^-$, dimer (m/z at 599 for $M\text{-}3H_2O{-}H^+)^-$, and monomer (m/z at 308 for $M\text{-}3H_2O{-}H^+)^-$ fragments (see Fig. 4*A*), the fragmentation pattern for the reaction product was slightly different, probably due to the presence of alternating α2,8- and α2,9-linkages in the cell-free reaction product(s). The degree of polymerization appears to be limited by the concentration of CMP-NeuAc. The production of a pentamer as the major product in the cell-free reaction is probably due to the limitation of CMP-NeuAc concentration along with the limited degree of polymerization. Increasing the amount of CMP-NeuAc in the cell-free reaction indeed increases the chain length of the sialyl polymer as judged by mass spectrometry (Fig. 5). While the MALDI-MS (Core Facility, The Scripps Research Institute) of the commercially available pentamer (Calbiochem) showed a peak for m/z at 1473, a peak at m/z 1474 was obtained with 10 mM CMP-NeuAc and at m/z 3830 with 20–30 mM CMP-NeuAc.

The *E. coli* strain K1 which forms the homopolymer of α2,8-linked sialic acids in its capsule was transformed with the *neuS* gene from K92. This was done to investigate whether the K92 *neuS* gene product competes with the endogenous α2,8-polysialyltransferase for the same endogenous acceptor *in vivo*. The capsular polysaccharide from the transformed K1 was isolated and purified by Bio-Gel P-2 column chromatography as mentioned under "Materials and Methods." ^{13}C NMR of this polysaccharide in D_2O showed a spectrum different from that of the K1- and K92-derived polysialic acids (Fig. 6). The major peaks for the $NCOCH_3$ and $NCOCH_3$ were similar to those of both K1 and K92. However, the signal for C-2 at δ103.44 was comparatively weaker. This may be due to the complex nature of the polysaccharide from transformed cells. Moreover, the ^{13}C NMR spectra obtained for the transformed K1 was similar to that of the polymer obtained from the K92 strain of *E. coli*, which also showed two closely adjacent signals at δ 23.36 and

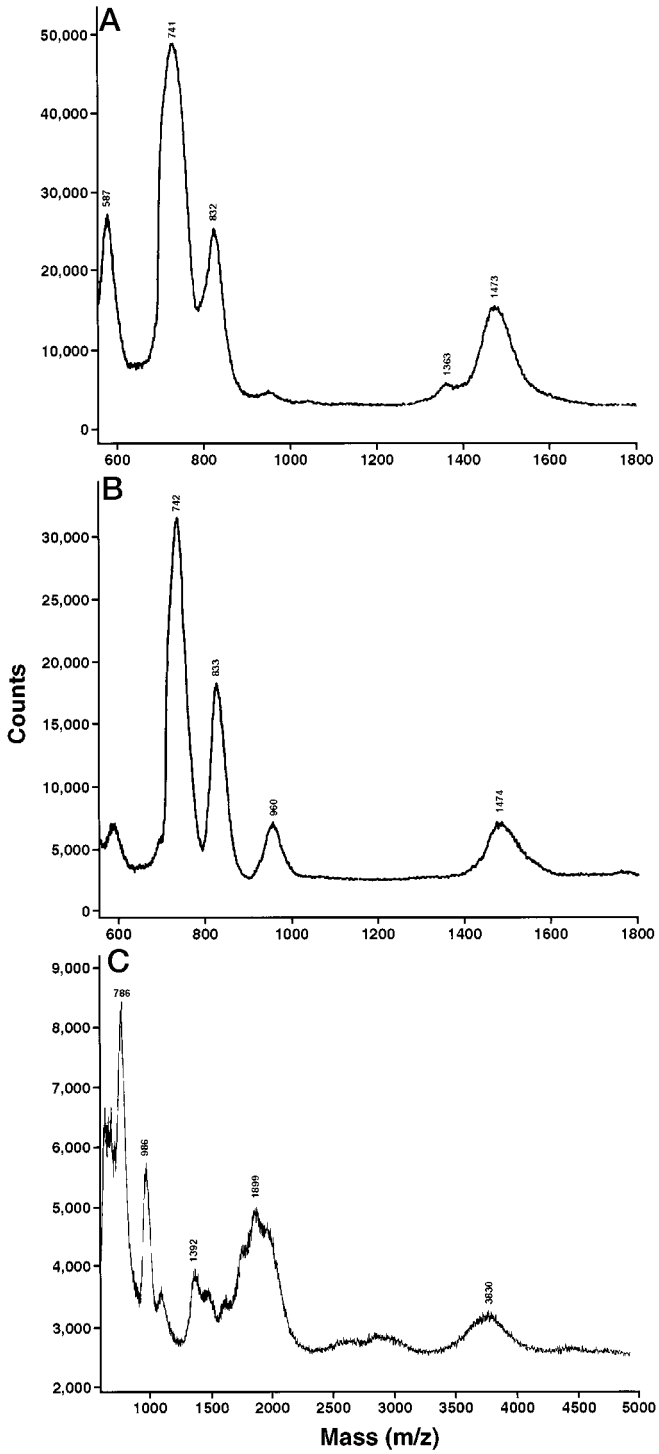

FIG. 5. **Mass spectral analysis of the enzyme reaction products with increasing concentration of CMP-NeuAc.** The cell-free enzyme reactions were performed as described in the legend to Fig. 4 with 1 mM sialic acid and increasing concentration of CMP-NeuAc (10, 15, 20, and 30 mM). After the reaction was complete, the materials were filtered (Amicon; 10 MWCO) and the filtrate from each reaction tube was directly used for mass-spectrometry (MALDI-MS). The spectra were: *A*, standard pentamer (Calbiochem); *B*, reaction product using 10 mM CMP-NeuAc; and *C*, reaction product using 20 mM CMP-NeuAc. The major peaks for *m/z* were shown by the *arrow* and described in the text. A similar spectrum (as *C*) was observed using 30 mM CMP-NeuAc.

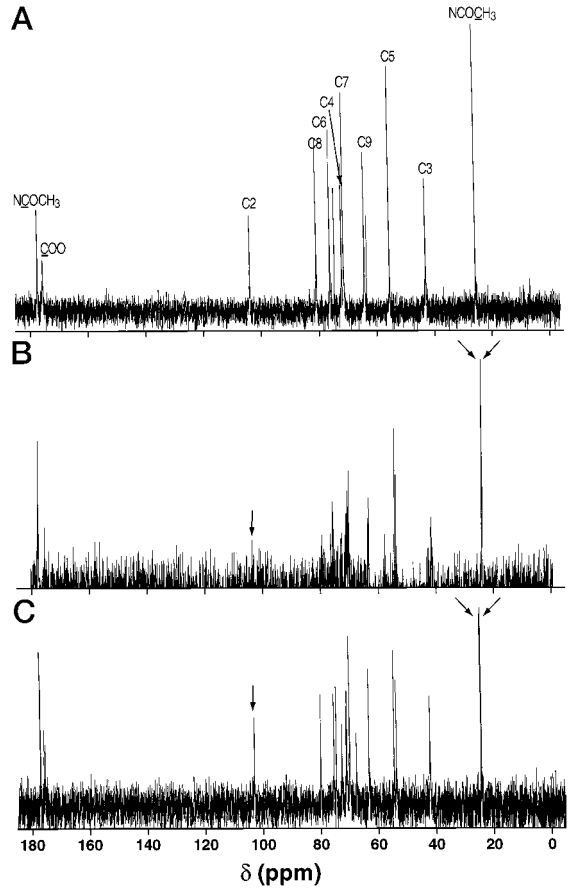

FIG. 6. **^{13}C NMR analysis of the capsular polysaccharide.** The spectra were recorded on a Brucker DRX-600 spectrometer. The scale is in parts per million and relative to external dimethyl sulfoxide. The spectra of the purified capsular polysaccharies were from K1 (*A*), K1 transformed with K92*neuS* (*B*), and K92 (*C*). The numbering of the carbon atoms is based on the previously published results (10).

23.51. It was shown earlier that this particular strain of *E. coli* contains heteropolysialyl oligosaccharide with alternating α2,8/2,9-linkages (10). This complexity was also evident in the ^{1}H NMR spectrum (Fig. 7) which showed two peaks at δ1.99 and δ1.96. While a detailed structural study is in progress, the preliminary data indicates that the polysaccharide isolated from the transformed K1 has both α2,8- and α2,9-linkages present in random sequences. This suggests that not only is the K92 *neuS* gene functional in the K1 strain of *E. coli*, but also that its expression disrupted normal polysialyl chain elongation. This result is probably due to the competition for the same endogenous acceptor *in vivo*. It also suggests that the endogenous acceptor is neither sialic acid nor colominic acid, but is common for both the α2,8- and α2,8/2,9-polysialyltransferases.

Interestingly, the cell-free enzyme reaction product(s) appears to be prone to lactone formation. As shown in Fig. 3, The radiolabeled product ran as a non-polar sialoside on DEAE-cellulose TLC and also passed through a column of Dowex 1X8 (phosphate form). Lactone formation appears to depend on sample preparation conditions, and seems to be preferable at lower pH. This lactonization was also observed at pH 8.0, presumably because the pH of the reaction mixture decreased during the reaction. The electrospray mass analysis also suggested lactone formation of cell-free reaction product(s). The peak for *m/z* at 1401 is consistent with the loss of four H_2O to form 4 lactones (Fig. 4) and the major peak for *m/z* at 1419 is consistent with the formation of 3 lactones from the pentameric product. Additional supporting evidence for the presence of lactones is the ^{13}C peaks at ~97 ppm, (Fig. 8), which are also observed in the authentic pentamer lactone prepared from the partial hydrolysis of the K92 polysaccharide. These peaks shift to ~103.5 ppm when lactone hydrolysis under basic conditions is performed. Based on the recent observation of regioselective

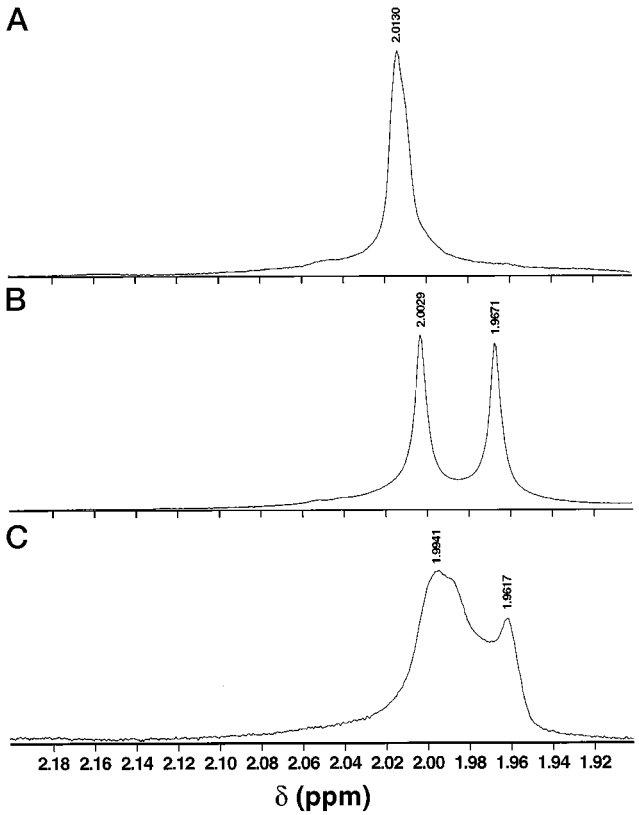

FIG. 7. **^{1}H NMR analysis of the capsular polysaccharide of K1 transformed with *K92/neuS*.** The spectra were recorded on a Brucker DRX-600 at an ambient temperature. Only the region for amide methyl groups from α2,8- and α2,9-linkages of the purified capsular polysaccharide have been shown for comparison. The isolation and purification of the capsular polysaccharides were described under "Materials and Methods." The spectra of the capsular polysaccharides were from K1 (*A*), K92 (*B*), and K1 transformed with K92/neuS (*C*). While the K1 sample showed only a singlet at δ1.99, K92 showed two peaks at δ2.00 and δ1.96. The spectrum obtained from the transformed K1 was broad and complex, especially at ~δ1.99.

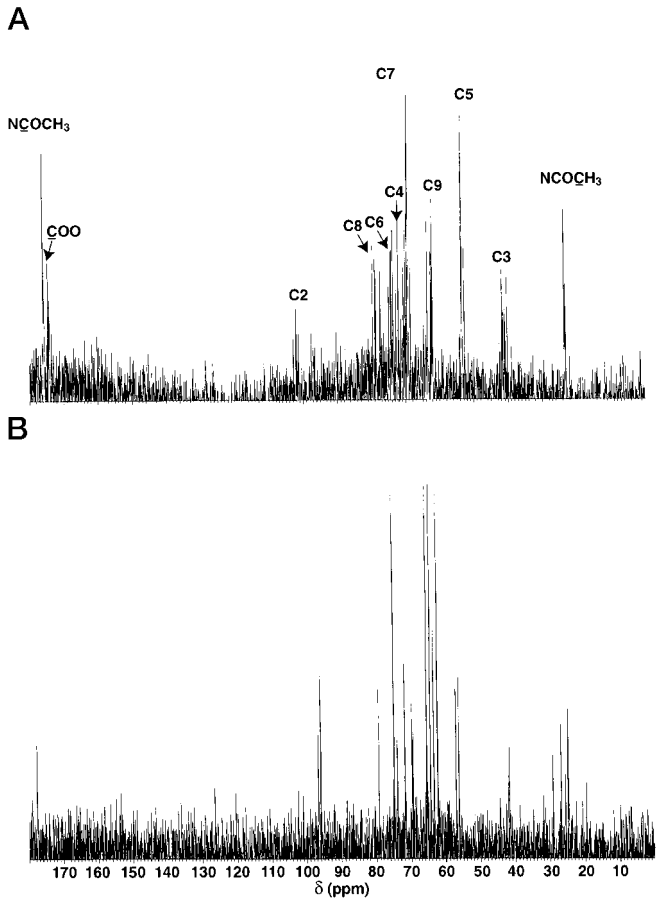

FIG. 8. **^{13}C NMR analysis of the enzyme reaction product.** After the enzyme reaction, the protein was removed by filtration (Amicon; 10,000). The enzyme reaction product was separated from unreacted sialic acid and CMP-NeuAc by passing through a column of Dowex 1X8 (PO_4^- form) eluting with phosphate buffer. The product was collected in the void volume and desalted by passing through a small column of Sephadex G-50 eluting with water. The desired material was collected in the void volume, freeze-dried, and dissolved in D_2O for NMR analysis. The spectra were recorded on a Brucker DRX-600 spectrometer at an ambient temperature (20 °C). The scale is in parts per million and relative to external dimethyl sulfoxide. Spectrum *A*, commercially available α2,8-linked pentameric sialic acid used as standard, results from approximately 25,000 accumulations. Spectrum *B*, partially purified cell-free reaction product, results from approximately 40,000 accumulations.

lactonization of α2,8-linked oligomeric sialic acid (33, 34), the carboxyl group of the nonreducing end sialic acid may exist as an open form (Fig. 9).

DISCUSSION

While sialic acid is ubiquitous in eukaryotes, particularly in mammals (12), it is not detected in prokaryotes other than some pathogenic bacteria. These include some *E. coli* strains, some *N. meningitidis* strains and bacteria of the O serotype including *Salmonella* (35–37). Although the presence of polysialyltransferase activity in *E. coli* was reported earlier by Roseman *et al.* (17), the nature of the gene and its product was not clearly demonstrated until recently. The cloned *neuS* gene from K1 showed that its product has similar size but no sequence homology to any of the mammalian sialyltransferases. Expression of the K1 *neuS* gene indicates that this 47-kDa protein synthesizes polysialic acid *in vitro* (13). A similar gene was cloned from the K92 strain of *E. coli*, the capsule of which was shown to contain heteropolysialic acid (-8NeuAcα2, 9NeuAcα2-) (10). This gene product differs by 70 amino acids when compared with that of the *neuS* gene product of the K1 strain (14). Of the observed amino acid replacements, 36 were conservative. On the basis of such homology, it was postulated that the K92 gene evolved from *neuS* of K1 (14). By complementation assay, the K92 *neuS* gene was shown by this group (23) to express polysialyltransferase activity yielding a product that was recognized by meningitidis C antiserum *in vivo*, suggesting that this gene might produce sialoside with alternating α2,8/2,9-polysialyl linkages. However, because meningitidis C antiserum interacts with both homopolysialic acid (-9-NeuAcα2-) from *N. meningitidis* Group C and heteropolysialic acid (-8Neu Acα2,9NeuAcα2-) from the K92 strain of *E. coli* (38), structural identification of the cell-free reaction product(s) was warranted. Previously, the expression of the K92 *neuS* gene was not clearly demonstrated, nor was it shown that this gene product catalyzes the synthesis of heteropolysialic acid (-8NeuAcα2,9NeuAcα2-) *in vitro*.

The polysialyltransferase encoded by *neuS* in *E. coli* uses CMP-sialic acid as a donor and catalyzes sequential sialylation at the 9-position of the nonreducing end of appropriate acceptors. Using K1 *neuS*, the polymerase activity was detected *in vitro* with sialyloligomers (designated colominic acid) or with certain gangliosides, such as G_{D3} (13) as substrates. However, it was shown previously that this gene product could not initiate *de novo* polysialic acid synthesis (23), indicating a requirement for an as yet undefined *in vivo* primer. This primer, or

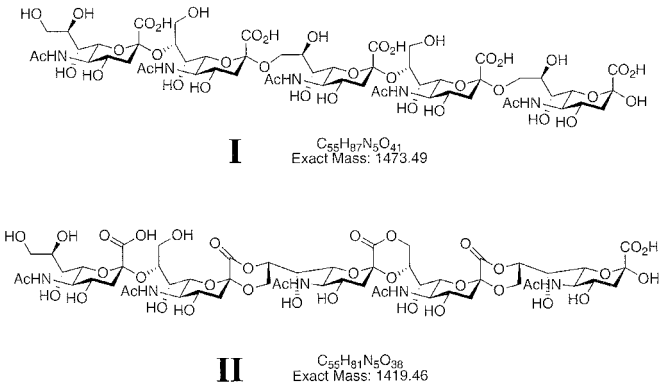

FIG. 9. **Proposed structure of the cell-free reaction product.** The product obtained by the cell-free reaction using K92 *neuS* protein, indicated the structure of a pentameric sialic acid with alternating α2,9/2,8-linkages (*I*). The proposed major lactone form (*II*) obtained in the enzymatic reaction is also shown.

polymerization initiator, does not appear to be colominic acid, since this polymer was neither elongated efficiently *in vitro* (18) nor the elongated product detected *in vivo* (39). Using K92 *neuS*, we have found that colominic acid is not an acceptor. In fact, among the acceptors tested, only sialic acid and certain gangliosides were found to be good acceptors. The real substrate specificity, however, remains to be investigated using the purified enzyme.

In conclusion, this report describes for the first time that the *neuS* gene product from *E. coli* K92 exhibits α2,8/2,9-polysialyltransferase activity both *in vitro* and *in vivo*. Apparently a single polypeptide (as judged by the Western blot) for this polysialyltransferase catalyzes the synthesis of polysialic acid with alternating α2,9- and α2,8-linkages. Previously, bacterial KDO-transferase was shown to possess such dual linkage specificity (40). Thus, enzymes capable of forming multiple different linkages may not be uncommon in nature. A recent report on the study of a crude preparation of polysialyltransferase from K92 indicates that the enzyme recognizes an α2,8-glycosidic linkage of sialic acid at the nonreducing end (41), consistent with our finding. It also indicates that the α2,9-linked homopolysialic acid or the α2,8/α2,9-linked heteropolymer of sialic acid with α2,9-linkage at the nonreducing end is not acceptable (41), and the enzyme may catalyze the sequential transfer of a preformed α2,8-linked dimer to the 9-position of the acceptor, a novel mechanism which remains to be proven. Work is in progress to purify the enzyme and to define the substrate specificity and mechanism.

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