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

CHARACTERIZATION OF THE ENZYME AND ITS REACTION PRODUCTS

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The capsular polysaccharide of *Escherichia coli* K92 contains alternating -8-NeuAc- and -9-NeuAc- linkages. The enzyme catalyzing this polymerization 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 hexamer 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-acetylenuraminic acid and the gangliosides G93 and GQ1b, 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 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 employed as a carbohydrate vaccine in the clinical trial of patients suffering from acute meningitis (11).

The sialyltransferases that catalyze the addition of sialic acid to form 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

by α2,8-, α2,9-, or α2,8α2,9-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).
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 \( a_{2,8} \)- and \( a_{2,9} \)-linkages in vitro and in vivo.

**MATERIALS AND METHODS**

**Cloning of 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. This was performed in a 100-μl reaction mixture containing 100 ng of genomic DNA as template, 300 nmol of primers, 200 μM dNTPs, 50 mM KCl, 10 μM Tris-HCl (pH 8.3), 2 mM MgCl2, 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 BamHI (underlined site) (5'-ATATTTAAGCTTACCACTGCTAGTCTTTTT-3'); nucleotides 3–20) and a reverse primer with EcoRI site, (5'-GGGGCAGATCTTACCTCCCCAAGAAA-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 1.4 kb. This fragment was purified by agarose gel electrophoresis, digested with BamHI and EcoRI, 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 sequence (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 forming gene in its chromosome and therefore does not require any pressure for the expression of its sex pilli. M13 phage containing the T7 forming gene in its chromosome, 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 forming gene in its chromosome (18), 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 forming gene in its chromosome (18), was used initially to express the gene.

**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 with the mouse anti-(His), monoclonal antibody (Qiagen, Valencia, CA) diluted 1:500 with the blocking buffer and 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 Virm 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 MgCl2, 5 mM dithiothreitol (DTT), and 1 μM polyalcohol oligosaccharide mixture (Sigma). The cells were lysed by freezing and thawing three times. A French Press (Amino; 1,500 p.s.i.) was used to remove 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 sonicated in a buffer containing 25 mM Tris-HCl, 5 mM MgCl2, 5 mM DTT, and 0.1% Triton CF-54. Other substrates were also used, including colominic acid. Both the endogenous and exogenous enzyme activity assays measure the transfer of 14C-labeled NeuAc to the acceptor. Unreacted radiolabeled substrates were separated by DEAE-cellulose TLC (1-propanol, 14 mM NH4OH/H2O, 6:1:3) or by Sephadex G-25 column chromatography. Radioactivity remaining at the origin and/or at the top of the TLC plate was cut out and the 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 \( A_{600} \sim 0.3 \) and 1 μM 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 resuspended in 0.9 mM CaCl2 and precipitated with 3 volumes of ethanol (−20 °C) absolute ethanol. The ethanol buffer (0.9 mM CaCl2) 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% in H2SO4, 80 °C), quantitation of sialic acid was transferred per hour.

**Thin-layer Chromatography and Fluorography—**

After the cell-free reaction was complete, the proteins were removed by an Amicon filter (MWCO 10,000), 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 mM NH4OH/H2O, 6:1:3; (b) chloroform, methanol, 0.02% CaCl2, 30:55:15. Control chromatograms were calibrated with mono- to hexa-sialosyl oligomers (Calbiochem, San Diego, CA) which were detected by orcinol spray, as described by Corfield and Schauer (25). This method resolved mono- to octa-sialosyl oligomers. Oligomers composed of sialic acid with more sialosyl residues were not retained at the origin. Labeled chromatograms were sprayed with En3Hance (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 reaction labeled reaction product (9-kDa) was marked after alignment of radioactive signals, cut and counted.

**Isolation and Purification of Polysialylated Oligosaccharides—**

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

**Protein Assay**—This was done using bicinonic 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 nL/min. The positive and negative ions, generated by charged droplet evaporation, enter the analyzer through an interface plate and a 100-mm orifice, where 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$_2$O, 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 NHSO (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, Vimm 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 StT6Gal 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 19 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.
The cell-free crude extract (15 K Sup and 40 K Sup) and the cell luncia, CA). The solvents used were: (a) 1-propanol, 1 M NH₄OH, water; 7:1:2; (b) chloroform, methanol, 0.02% CaCl₂; 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 G₃G₃ 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 NH₄OH, 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—For structural determination, the cell-free reactions were carried out, using the 40,000 supernatant as the enzyme source, though the fusion protein apparently binds to the Ni²⁺-NTA-agarose column failed. Although the fusion protein apparently binds to the Ni²⁺-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).

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-[¹⁴C]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-[¹⁴C]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. 3C). 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 2,8-linkages were not accepted, whereas the corresponding dimer and trimer (Calbiochem) served as poor acceptors. Among the glycolipids tested, GD3 and GQ1b also served as good acceptors. However, other sialylgangliosides tested were poor acceptors. Notably, pentameric and hexameric polysialosides with 2,8-linkages were not accepted, whereas the corresponding dimer and trimer (Calbiochem) served as poor acceptors. Among the glycolipids tested, GD3 and GQ1b also served as good acceptors. However, other sialylgangliosides tested were poor acceptors (Table I).

**Table I**

| Potential acceptor substrates tested | Counts/min obtained per 60-μl reaction | Total sialic acid transferred 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                                |
| GD₃                                 | 2850                                  | 360                              |
| GM₃                                 | 1440                                  | 190                              |
| GD₃                                 | 900                                   | 110                              |
| G₄T₄rong                            | 460                                   | 40                               |
| G₃T₃rong                            | 1140                                  | 130                              |
| G₃Q₄                                | 2460                                  | 310                              |
| Ganglioside mixtures                | 720                                   | 90                               |

The reaction mixture (60 μl) contained 1 μM each of the following acceptors and 9 nmol of CMP-[¹⁴C]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 NH₄OH; 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.

**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-[¹⁴C]NeuAc (9 nmol; 1 μCi) as donor in a reaction buffer containing 25 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 5 mM DTT, and 0.5% Triton CF-54. As an example, analysis was shown with the reaction mixture containing sialic acid and G₃G₃ as acceptors and CMP-[¹⁴C]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), G₃G₃ 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 NH₄OH, water; 7:1:2; (b) chloroform, methanol, 0.02% CaCl₂; 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 G₃G₃ as acceptor (lane 5) and after acidification (lane 6); reaction mixture without exogenous acceptor (lane 7) and after acidification (lane 8). The area corresponding to the reaction product was clearly showed the presence of a single band at about 47.5 kDa (Fig. 2). The α2,8/2,9-polyosialyltransferase was also found to be present in the 40,000 supernatant. Our initial attempt to purify the protein using Ni²⁺-NTA-agarose column failed. Although the fusion protein apparently binds to the Ni²⁺-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).
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 $(M-3H \cdot 2O \cdot H) \cdot 2$ and at $m/z$ 1401 for $(M-4 H2O \cdot 2H)$. This spectrum was similar to that of the commercially obtained $\alpha_{2,8}$-polysialic acid (Calbiochem). While the authentic sample showed a fragmentation pattern accounting for the tetramer $(m/z$ at 1182 for $M-3H2O \cdot H) \cdot 2$, trimer $(m/z$ at 890 for $M-3H2O \cdot H) \cdot 2$, dimer $(m/z$ at 589 for $M-3H2O \cdot H) \cdot 2$, and monomer $(m/z$ at 308 for $M-3H2O \cdot H) \cdot 2$ fragments (see Fig. 4A), the fragmentation pattern for the reaction product was slightly different, probably due to the presence of alternating $\alpha_{2,8}$- and $\alpha_{2,9}$-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 $\alpha_{2,8}$-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 $\alpha_{2,8}$-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$_2$O showed a spectrum different from that of the K1- and K92-derived polysialic acids (Fig. 6). The major peaks for the NCO$^-$C$^3$H$_3$ and N$^-$C$^3$OCH$_3$ were similar to those of both K1 and K92. However, the signal for C-2 at $\delta$ 103.44 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 $\delta$ 23.36 and

![Fig. 4. Mass-spectral analysis of the enzyme reaction product.](http://www.jbc.org/Downloaded from)
It was shown earlier that this particular strain of E. coli contains heteropolysialyl oligosaccharide with alternating $\alpha_{2,8/2,9}$-linkages (10). This complexity was also evident in the $^1$H NMR spectrum (Fig. 7) which showed two peaks at $\delta_{1.99}$ and $\delta_{1.96}$. While a detailed structural study is in progress, the preliminary data indicates that the polysaccharide isolated from the transformed K1 has both $\alpha_{2,8}$- and $\alpha_{2,9}$-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 \emph{in vivo}. It also suggests that the endogenous acceptor is neither sialic acid nor colominic acid, but is common for both the $\alpha_{2,8}$- and $\alpha_{2,8/2,9}$-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 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.

![Mass spectral analysis of the enzyme reaction products with increasing concentration of CMP-NeuAc](image)

**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.
lactonization of α,2,8-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 (α2,8NeuAc2,9NeuAc2-) (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 α,2,8/2,9-polysialyl linkages. However, because meningitidis C antiserum interacts with both homopolysialic acid (9-NeuAcα2-) from N. meningitidis Group C and heteropolysialic acid (α2,8NeuAcα2,9NeuAcα2-) 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 (α2,8NeuAcα2,9NeuAcα2-) 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 sialylligomers (designated colominic acid) or with certain gangliosides, such as G2 (33) 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
The product obtained by the cell-free reaction using K92 indicated the structure of a pentameric sialic acid with alternating 2,9-linkages may not be uncommon in nature. A recent report on the acceptor specificity (40). Thus, enzymes capable of forming multiple different substrate specificity and mechanism.

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 _vivo_ (18) nor the elongated product detected in _vitro_ (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 2,8/2,9-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 2,9- and 2,8-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 2,8-glycosidic linkage of sialic acid at the nonreducing end (41), consistent with our finding. It also indicates that the 2,9-linked homopolysialic acid or the 2,8α2,9-linked heteropolymer of sialic acid with 2,9-linkage at the nonreducing end is not acceptable (41), and the enzyme may catalyze the sequential transfer of a preformed 2,8-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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