Successive glycosyltransfer of sialic acid by *Escherichia coli* K92 polysialyltransferase in elongation of oligosialic acceptors

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*Escherichia coli* K92 produces a capsular polysialic acid with alternating α2,8 α2,9 NeuNAc linkages. This polysaccharide is cross-reactive with the neuroinvasive pathogen *Neisseria meningitidis* Group C. The K92 polysialyltransferase (PST) catalyzes the synthesis of the polysialic acid with alternating linkages by the transfer of NeuNAc from CMP-NeuNAc to the nonreducing end of the growing polymer. We used a fluorescent-based high-performance liquid chromatography assay to characterize the process of chain extension. The PST elongates the acceptor GT3-FCHASE in a biphasic fashion. The initial phase polymers are characterized by accumulation of product containing 1–8 additional sialic acid residues. This phase is followed by a very rapid formation of high-molecular weight (MW) polymer as the accumulated oligosaccharides containing 8–10 sialic acids are consumed. The high-MW polymer contains 90–100 sialic acids and is sensitive to degradation by periodate and K1–5 end neuraminidase, suggesting that the polymer contains the alternating structure. The polymerization reaction does not appear to be strictly processive, since oligosaccharides of each intermediate size were detected before accumulation of high-molecular weight polymer. Synthesis can be blocked by CMP-9-azido-NeuNAc. These results suggest that the K92 PST forms both α2,8 and α2,9 linkages in a successive and nonprocessive fashion.

**Key words:** capsular polysaccharide/chain extension/polysialyltransferase/processivity/sialic acid

Introduction

Polysialic acids play numerous roles in cellular interactions. In pathogenic bacteria, these acidic polysaccharides serve as extracellular shields against the defense systems of their mammalian host. Human pathogens encapsulated with polysialic acids cause invasive disease such as meningitis and urinary tract infection (Jann et al. 1992). There are three polysialic acid homopolymerides identified in Nature, α2,8 NeuNAc (McGuire et al. 1964), α2,9 NeuNAc (Bhattacharjee et al. 1975), and the alternating structure α2,8 α2,9 NeuNAc (Egan et al. 1977). *Escherichia coli* K92 produces the alternating polysialic acid and is serologically cross-reactive with the α2,9 NeuNAc capsular polysaccharide of the neuroinvasive pathogen *Neisseria meningitidis* Group C (Egan et al. 1977).

The genetic loci encoding the expression of bacterial polysialic acid have been well characterized (Whitfield and Roberts 1999). A single polysialyltransferase (PST)1 has been identified in the gene clusters of *E. coli* and *Neisseria* that produce polysialic acid capsule (Steenbergen et al. 1990). The K1 and K92 PSTs are membrane-bound enzymes and require the addition of an acceptor molecule to measure enzyme activity. These PSTs elongate the growing chains of polysialic acid from the nonreducing end. They transfer NeuNAc from CMP-β-NeuNAc with inversion of the anomic configuration and belong to the CAZY glycosyltransferase family 38 (Coutinho et al. 2003). The sequence-based family 38 glycosyltransferases consist entirely of the PSTs of *E. coli* and *Neisseria*. These PSTs are therefore expected to have different 3D structures from the recently reported sialyltransferase CSTII of *Campylobacter* (Chiu et al. 2004) belonging to family 42 (Coutinho et al. 2003). The enzymes in family 38 have not been purified and the mechanism of polymerization has not been characterized in detail.

We reported that the K92 PST can elongate all known polysialic acids and oligosialic acids when PST is incubated with these polymers in vitro as acceptors (McGowen et al. 2001). In vivo, the K92 PST is responsible for synthesis of both α2,8 and α2,9-linked NeuNAc. Our investigation of the acceptor specificity suggests that the PST prefers oligosaccharide acceptors with a hydrophobic aglycon. Although the PST does not elongate distialylogosaccharides, it does elongate gangliosides possessing a NeuNAc α2,8 NeuNAc and the fluorescent ganglioside analog GD3-FCHASE (McGowen et al. 2001). In this report, we take advantage of these fluorescent ganglioside analogs as acceptors to characterize the K92 PST elongation reaction.

Polysaccharides are often degraded by processive enzymes (Horn et al. 2006). These enzymes bind to the polymer and perform multiple cleavages without dissociating. Chitin and cellulose degradation by processive enzymes is indicated by the pattern of accumulation of degradation products (Mello et al. 2002; Soerbotten et al. 2005; Sikorski et al. 2006). In a similar manner, the pattern of accumulation of products has been used to examine the processivity of plant and bacterial glycosyltransferases (Akita et al. 2002; Guillaumie et al. 2003; Forsee et al. 2006). The type 3 capsular polysaccharide of *Streptococcus pneumoniae* is synthesized by a processive glycosyltransferase (Forsee et al. 2006). A critical oligosaccharide length of approximately eight monosaccharides is required for recognition of the growing chain by the

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glycosyltransferase. Upon binding of the oligosaccharide–

lipid acceptor to the carbohydrate recognition site of the glycosyltransferase, the polymerization reaction enters a

highly processive phase to produce polymer of high molecular weight (MW). In the experiments described later, we show that the K92 PST elongates the trisialyl acceptor, GT3-FCHASE, rapidly with release of each intermediate from the enzyme suggesting a non-processive process.

Results

Earlier reports have suggested that the K1 PST can extend short oligosaccharides of sialic acid by only a few residues (Steenbergen et al. 1990; Ferrero et al. 1991). Cho and Troy (1994) showed that K1 PST can extend disialylgangliosides to polymeric material. Our laboratory has shown previously, that the K92 PST can elongate disialylgangliosides (McGowen et al. 2001). We demonstrated that a fluorescent analog of GD3 acts as an acceptor (primer) for K92 PST (McGowen et al. 2001). In the current report, we performed a more detailed analysis of the K92 elongation reaction. We took advantage of the fluorescent primer GT3-FCHASE to characterize the primer extension reaction of K92 PST. We wanted to determine whether in vitro extension of oligosialic acid consists of the addition of a few residues or the formation of high-MW polymer, and to determine whether the product is an alternating polymer. Since the degree of processivity of this reaction is unclear, we investigated the mode of polysialic acid chain elongation by K92 PST.

The trisialylated oligosaccharide GT3-FCHASE was incubated with membranes harboring the K92 NeuS for 30 min and the soluble product contained in the high-speed supernatant was analyzed by ion exchange high-performance liquid chromatography (HPLC). As is shown in Figure 1, GT3-FCHASE was completely consumed and converted to a major species eluting near the end of the ion exchange gradient. This suggested that the fluorescent oligosaccharide had been converted into a high-MW polymer. We isolated this late eluting species and treated it with either sodium periodate or the α2,8-specific K1-5 endoneuraminidase to demonstrate that this peak was indeed a polysialic acid. The results in Figure 2 show that this late eluting species is sensitive to both treatments. Treatment of the isolated species with sodium periodate resulted in a marked decrease in the peak at 100 min. That polysaccharide chains were cleaved by periodate was confirmed in a separate experiment by oxidation of the high-MW peak and reduction with NaB3H4. A degradation product similar to that expected for oxidized sialic acid was detected in a hydrolysate of the oxidized and reduced high-MW fraction (data not shown). Characteristically the K1-5 endoneuraminidase degrades the high-MW species to oligomers of sialic acid. The K1-5 endoneuraminidase is specific for α2,8 NeuNAc linkages and does not cleave α2,9 linkages. This result suggests that this late eluting species is a polysialic acid containing both α2,8 and α2,9-linked NeuNAc.

Nakata and Troy (2005) described a method for determining the chain length of polysialic acids in cell extracts based on the elution position of the oligosialic and polysialic acid on an ion exchange gradient. We used the ion exchange gradient described for this method to estimate the chain length of polysialic acid resulting from the extension of GT3-FCHASE after a 30-min reaction. As is shown in Figure 3, the product elutes at a position expected for polysialic acids with a degree of polymerization of 90–100 sialic acid residues. Polysialic acid formed after a 30-min incubation eluted as a single peak in the 90–100 degree of polymerization (DP) range. However, the polysialic acid formed after a 5 min reaction is smaller and more polydisperse than that formed after a 30 min reaction, suggesting that at earlier time points smaller size oligosialic acids are formed that are eventually elongated to the 90–100 DP range.

Time course of GT3-FCHASE extension

It appears from the experiments described in Figure 1, that the elongation reaction is very rapid. Expansion of the early portion of the chromatogram in Figure 1 suggests that oligosialic acid intermediates are formed during the polymerization reaction. We developed an assay to detect the early stages of the polymerization reaction and to determine if oligosialic acid intermediates are formed. We used ethanol to inactivate the enzyme and stop the reaction. Oligosialic acid intermediates were not detected in reaction mixtures containing the K1 PST of E. coli strain K235 by the radiolabel assay. Similarly, oligosialic acid intermediates were not detected in in vivo pulse reactions of E. coli K1 PST (Steenbergen and
This result has been interpreted by previous authors to suggest that the enzyme might polymerize sialic acid in a processive reaction.

Oligosialic acids were observed immediately after initiation of extension of GT3-FCHASE. The chromatograms in Figure 4 show that oligosialic acids of increasing size are observed ranging from elongation of GT3-FCHASE by a few sialic acids to the high-MW polysialic acid. The size of the high-MW polysialic acid peak increases as the concentration of the low- and intermediate-MW oligosialic acids decreases, suggesting that oligosialic acid is consumed in the formation of polysialic acid. This suggests that the K92 PST is not strictly processive but allows the dissociation of the product after each addition of sialic acid to GT3-FCHASE. To confirm that the dissociated intermediates are being consumed and converted into polysialic acid we isolated several peaks from scaled up 2 min reactions. The purified intermediates were incubated in a second reaction with K92 PST for 5 min.

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and applied to the ion exchange column. In Figure 5 are shown the chromatograms of these peaks, illustrating their conversion to polysialic acid after the second incubation.

The K92 PST also appears to have a preference for higher DP oligosialic acids. In Figure 4A and B at early time points there is a greater accumulation of oligosialic acids with a DP less than 10–12. Oligosialic acids with higher DP are in lower concentration and appear to be converted more rapidly into polysialic acid. With time, the lower DP oligosialic acid appears to be converted to higher DP material since the concentration of the lower DP species decreases as the concentration of the high DP species increases (Figure 4). We interpret these results to mean that the PST has a higher affinity for the higher DP polysialic acid.

Dependence of GT3-FCHASE extension on substrate concentration

The size distribution of reaction products of the K92 PST is very dependent on CMP-NeuNAc concentration. At 250 ìM CMP-NeuNAc the major species formed is high-MW polymer. However, at low concentrations (5–50 ìM) the predominant products represent the addition of only a few sialic acids to GT3-FCHASE. This concentration range (5–50 ìM) is well below the reported $K_m$ for CMP-NeuNAc, 250–300 ìM, (Ortiz 1989) (data not shown). We tested the ability of nascent GT3-FCHASE products to remain bound to the membrane complex after washing. We could not detect significant amounts of fluorescent product bound to K92 membranes after washing to remove substrates. These results support the conclusion that K92 PST does not elongate acceptors in vitro in a processive fashion.

Chain termination of GT3-FCHASE extension reaction

The K92 PST appears to be adding sialic acid in both 2,8 and 2,9 linkages based on the results described in Figure 2. This result implies that inclusion of appropriate substrate analogs would limit the growth of the polysaccharide chain by termination of GT3-FCHASE extension. As outlined in Scheme 1, when CMP-9-N3-NeuNAc is used as a substrate the transfer of sialic acid to the position-9 of the primer molecule occurs only once, thus blocking synthesis. We tested this hypothesis using the radiolabeled assay. Membranes of the sialic acid-negative strain construct, EV239:pWV213, were first incubated with either CMP-NeuNAc or CMP-9-N3-NeuNAc, then centrifuged and washed prior to the addition of radiolabeled substrate. As is shown in Table I, incubation with the 9-azido derivative inhibits incorporation of NeuNAc into

Scheme 1. Reaction of K92 polysialyltransferase with CMP-9-azidoNeuNAc.

<table>
<thead>
<tr>
<th>Membrane pretreatment</th>
<th>Amount (nmols)</th>
<th>CPM</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>16852</td>
</tr>
<tr>
<td>Buffer</td>
<td>—</td>
<td>16882</td>
</tr>
<tr>
<td>CMP-NeuNAc</td>
<td>15</td>
<td>7011</td>
</tr>
<tr>
<td>9-azido CMP-NeuNAc</td>
<td>15</td>
<td>1616</td>
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</tbody>
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Membranes prepared from EV239:pWV213 were incubated with 15 nmol substrate in 50 mM Tris, 25 mM MgCl2, pH 8.0 for 15 min. then diluted with 400 ìL of same buffer. The membranes were recovered by ultracentrifugation at 100 000 g for 30 min and resuspended in 50 ìL of buffer. The treated membranes were then assayed for the incorporation of $^{14}$C-NeuNAc by the polysialyltransferase activity assay described in the Materials and methods section. CPM, counts per minute.
polymer. Similarly, K92 PST activity was inhibited when CMP-9-N3-NeuNAc was added directly to assay mixtures.

If sialic acid is transferred in an alternating fashion then extension of GT3-FCHASE with CMP-9-azido-NeuNAc would result in synthesis of a truncated product. In Figure 6A, the product of CMP-9-N2-NeuNAc incubation with K92 PST and GT3-FCHASE eluted as a single peak at a position expected for the addition of 1–2 sialic acids to GT3-FCHASE. No species of higher DP are observed in this reaction mixture. That this observation is not due to simple inhibition was demonstrated by isolation of the CMP-9-N2-NeuNAc extended product and incubation with CMP-NeuNAc and active membranes. Again, the isolated product was not extended (Figure 6C). Products of similar DP isolated from CMP-NeuNAc containing reaction mixtures were converted to polysialic acid of the 90–100 DP during an equivalent time period (Figure 5).

Reaction mixtures containing both substrates, CMP-NeuNAc and CMP-9-N2-NeuNAc, resulted in apparent lower DP products. In Figure 7, the K92 PST was incubated with CMP-NeuNAc and CMP-9-N2-NeuNAc in a ratio of 1:1 and 1:5. A more extensive accumulation of oligosialic acids of lower DP was observed with the 1:1 mixture compared with the 1:5 mixture. Note that very little polysialic acid is formed even after extensive incubation of the reaction mixtures. This result suggests that the length of GT3-FCHASE chain extensions can be controlled by the ratio of modified substrate. These reagents are potentially useful tools for preparing tagged oligosialic acids of defined DP.

Discussion

The mechanism of chain extension by the E. coli PSTs is unclear from previous reports. It was concluded, based on the ability to extend a short oligosialic acid by a few residues, that the K1 PST catalyzes the successive addition of single sialic acid residues (Steenbergen and Vimr. 1990; Ferrero Fig. 7. Chain termination of the GT3-FCHASE extension reaction. K92 polysialyltransferase was incubated with 0.225 μM GT3-FCHASE and CMP-NeuNAc and 250 μM CMP-9-azidoNeuNAc at (A) a ratio of 1:5 (50 μM CMP-NeuNAc) or (B) a ratio of 1:1 for 30 min prior to adjusting the reaction mixtures to 25% ethanol. The supernatants were applied to a DNA Pac PA-100 column and chromatographed with a gradient of NaNO₃ (Inoue et al. 2001; Inoue and Inoue 2003).

Fig. 6. Chain termination of the GT3-FCHASE extension reaction. (A) K92 polysialyltransferase was incubated with 250 μM CMP-9-azidoNeuNAc and 0.225 μM GT3-FCHASE for 30 min. (B) The product from the DNA Pac PA100 column was isolated, and (C) incubated with CMP-NeuNAc for 30 min prior to injection and chromatographed with a gradient of NaNO₃ (Inoue et al. 2001; Inoue and Inoue 2003).
et al. 1991). The thin layer chromatography (TLC) method used in these experiments was not very sensitive. Furthermore, it appeared from these experiments that high-MW polymer was not formed from these oligosaccharidic acid acceptors, thus leaving in question whether these experiments reflected the mechanism in vivo. Chao et al. (1999) studied the elongation of K92 oligosaccharides by the K92 PST and concluded that the enzyme extends the growing chain by the addition of a disialic acid unit to the nonreducing end of the growing chain (Chao et al. 1999). In contrast, the time course of in vivo synthesis of K1 polysialic acid was measured by continuous pulse of the E. coli strain EV138 (nanA4, neuB25) culture exogenously with radioactive NeuNAc (Steenbergen and Vimr 2003). In this latter experiment no intermediate oligosaccharides were detected by TLC. The authors therefore concluded that the in vivo reaction occurs in a processive manner (Steenbergen and Vimr 2003).

In this report, we addressed the question of extension of polysialic acid chains using a very sensitive method based on a fluoroscent acceptor. This method has greater sensitivity than the radiolabel TLC method and has a high resolution. The properties of the assay enabled us to follow the extension reaction further than previously reported. Using this approach, we could show that the K92 PST extends its acceptor to form high-MW polymer in a rapid fashion. Furthermore, since intermediates of every size were detected, the addition of sialic acid to the growing chain probably occurs in a successive manner. This successive addition is further supported by the apparent conversion of intermediate oligosaccharides to polysialic acids of 90–100 DP. This result is not consistent with the processive dimer model or the in vivo processive model. Our observation that the high DP oligosaccharides are consumed rapidly would explain the inability to detect intermediates in the relatively insensitive in vivo TLC method. Oligosaccharide intermediates, if rapidly consumed, would be in low concentrations and below the limits of detection of TLC autoradiographs.

An alternative explanation is that the PST alone is nonprocessive and catalyzes successive additions. Most of the experiments in this report were done with membranes harboring NeuS and lacking other gene products of the K92 gene cluster. However, in the presence of an accessory protein from the K92 gene cluster the NeuS PST would become processive. This would explain the discrepancy between the interpretation of the in vivo and in vitro observations. One should also keep in mind that the results reported here were obtained with an artificial acceptor. Determination of how closely these experiments mimic in vivo synthesis awaits the identification and characterization of the natural acceptor.

The K92 PST elongated disialyl and trisialyloligosaccharides possessing a hydrophobic aglycon. This property was demonstrated for disialylgangliosides and the ganglioside analogs GD3-FCHASE and GT3-FCHASE. In previous experiments, transfer was not observed with disialyl or trisialyl acceptors lacking the hydrophobic aglycon. The sialyltransferase, CSTII, of Campylobacter jejuni transfers sialic acid to oligosaccharide acceptors attached to a hydrophobic residue. This is due to the fortuitous location of a hydrophobic region near the sugar acceptor binding site (Chiu et al. 2004). A similar situation could exist with the K92 PST.

The fact that the product dissociated from the enzyme after transfer of each sialic acid implies that specificity of transfer is influenced by the structure of the acceptor. After transfer of sialic acid to form a 2–9 linkage, this product dissociates and competes with oligosaccharides having 2,8 NeuNAc at the nonreducing end of the chain. In order to maintain the synthesis of an alternating 2,8-NeuNAc–2,9-NeuNAc structure, transfer must be to the 2,9 hydroxyl when the 2,8NeuNAc is bound and similarly, transfer must be to the 2,8 hydroxyl when the oligosaccharide end in a 2,9 linkage is bound. This observation rules out the two active site model proposed in our earlier publication (McGowen et al. 2001). Nevertheless the orientation of the 8- or 9- hydroxyl of sialic acid relative to the sugar nucleotide could dictate specificity of the enzymatic transfer. This question may be answered when crystallographic data are available for this enzyme.

CMP-9-N3-NeuNAc is a useful substrate to investigate the polymerization of the alternating polysialic acid structure. Attempts to use the 9-fluoroceinyl-NeuNAc derivative were unsuccessful since this analog is a poor substrate for the PST (Vann, unpublished results). CMP-9-N3-NeuNAc is a much better substrate, probably due to the relatively small size of the azido function. The use of this substrate blocks synthesis as predicted for alternating linkages.

Polysialic acids play important roles in normal cell function and disease. These polysaccharides are used as vaccines against infectious bacteria and markers of tumor development. Sensitive reagents for monitoring functions of polysialic acid would improve the therapeutic and diagnostic value of these polysaccharides. Overall the results in this report illustrate a path to the preparation of polysialic acids with selectively functionalized end groups. Jing and DeAngelis (2004) have also used a chemoenzymatic approach to prepare monodisperse polymer with tags at the reducing end of the medically important hyaluronic acid. Introduction of the azido group at the non-reducing terminus provides a means of controlling the length of the growing chain. In addition, it provides a site for chemically introducing probes or conjugating to proteins. 1,3 Dipolar cycloaddition of alkynes to azido is a mild and powerful route for attachment of oligosaccharides to microarrays, synthesis of multivalent neoglycoconjugates, and labeling of proteins and cell surfaces. The availability of this variety of defined reagents will facilitate characterization of antibody-antigen interaction, synthesis of vaccines, and characterization of cell interactions with polysialic acids.

Materials and methods
Bacterial strains and plasmids

The bacterial strains and plasmids used in this study have been described (Andreasheva and Vann 2006). E. coli K1 hybrid strain EV239 (neuS::Tn10 nanA4, neuB25) was obtained from Dr. Eric Vimr (University of Illinois, Urbana). The plasmid pWV213 contains the K92 neuS gene and was described previously (McGowen et al. 2001).

CMP-9-N3-NeuNAc synthesis

9-Azido-NeuNAc was synthesized according to Han et al (2005). CMP-9-N3-sialic acid was synthesized according to Shames et al. (1991) with a few modifications. The synthesis reaction consisted of 100 mg of CTP (Roche Applied Science, Indianapolis, IN) and 50 mg of 9-azido-sialic acid.
in 5 mL of 50 mM Tris, 50 mM MgCl₂, pH 8.5. The reaction was initiated by the addition of six units of E. coli CMP-NeuNAc synthetase and incubated at room temperature for
5 h with periodic removal of precipitate by centrifugation. The progress of the reaction was monitored by thiobarbituric acid (Vann et al. 1987). An additional 6 U of enzyme was
added for 1 h incubation. CMP-9-N₃-NeuNAc was precipitated
acid (Vann et al. 1987). An additional 6 U of enzyme was
The progress of the reaction was monitored by thiobarbituric
5 h with periodic removal of precipitate by centrifugation.
NeuNAc synthetase and incubated at room temperature for
was initiated by the addition of six units of
PST activity was assayed as described previously (McGowen
Polysialyltransferase activity
PST polymerization assay mixtures in the presence or absence
of CMP-NeuNAc as described in the text (Andreishcheva and Vann 2006)
Endon neuraminidase treatment
Reaction mixtures (25 µL) prepared for the PST polymerization
assay were incubated for 1 h prior to the addition of either 35 µg freshly purified K1-5 endon neuraminidase (Andreishcheva and Vann 2006) in 50 µL or 50 µL of
buffer as a control. The digestion mixtures were incubated at
37°C for 1 h, centrifuged and the supernatant analyzed on the DNA Pac column as described in the Polysialyltransferase
polymerization assay section. Alternatively, individual peaks from the PST reaction were
collected, purified by Sep-Pak, and redissolved with 50 µL
H₂O prior to digestion with endon neuraminidase. A 25 µL aliquot was incubated at 37°C for 10 min with 10 µL of
freshly purified endon neuraminidase, whereas the other 25 µL

Degree of polymerization
The DP of the high-MW GT3-FCHASE product was estimated using the HPLC gradient described by Nakata and Troy (2005). The high-MW peak from the GT3-FCHASE reaction
described in the Polysialyltransferase polymerization assay
section was collected and purified on a Sep-Pak cartridge (Waters part no. 051910) prior to chromatography on this gradient. Sep-Pak cartridges were wetted with 20 mL of 100% acetonitrile followed by 20 mL of H₂O. Peaks isolated from
HPLC were immediately loaded onto the prepared Sep-Pak
cartridges and allowed to flow through by gravity. The
cartridge was washed with 4 mL of H₂O followed by elution with 3 mL of 50% acetonitrile. The elution fractions were
dried, taken up in water, and applied to a DNA Pac PA-100 column. The DNA Pac PA-100 column was eluted with a convex gradient of 0–2 M ammonium acetate. In this gradient, the concentration of ammonium acetate at 0, 5, 15, 20, 35, 55, 145, and 182.5 min was 0, 0, 20, 25, 32.5, 40, 62.5, and
100% of 2.0 M. The DP was estimated by comparing the elution position in the ammonium acetate gradient with the elution position of polysialic acids described by Nakata and Troy (2005).

Chain termination reactions
The extension of polysialic acid chains on GT3-FCHASE was
terminated by the inclusion of 250 µM CMP-9-N₃-NeuNAc in
PST polymerization assay mixtures in the presence or absence
of CMP-NeuNAc as described in the text (Andreishcheva and Vann 2006).
aliquot is incubated with buffer. The reactions are loaded directly onto the DNA Pac column for HPLC analysis.

**NaIO₄ treatment**

The reaction mixture contained 50 μL of membrane preparation; 250 μM CMP-NeuNac, 40 ng of GT3-FCHASE, 35 mM Tris, 17.5 mM MgCl₂, pH 8.0 in 100 μL and was incubated at 37°C for 1 h. The reaction mixture was stopped by adjusting to 25% ethanol, treated as described in the Endonucleamidase treatment section and analyzed by HPLC. A single late eluting peak of high-MW material was collected, purified by Sep-Pak, and redissolved with 450 μL H₂O. A 225 μL aliquot was mixed with 225 μL of either 100 mM NaIO₄ or 225 μL of H₂O and incubated for 20 h at room temperature. The reaction treated with NaIO₄ was quenched by adjusting to 200 mM glycerol. Both reactions were analyzed by HPLC.

To confirm the identity of the periodate degradation products by paper chromatography, the earlier PST reaction was scaled up to a 250 μL reaction. The high-MW peak isolated by HPLC and Sep-Pak cartridge was dissolved in 100 μL water and processed as follows. A 50 μL aliquot was mixed with 50 μL of either 10 mM NaIO₄ in 20 mM sodium acetate, pH 5.6 or 50 μL of 20 mM sodium acetate, pH 5.6, and incubated 2 h at 37°C. Both reactions were quenched by adjusting to 20 mM glycerol and reduced with 10 mM NaB₃H₄ (specific activity 22.6 Ci/mmol). The reactions were acidified with Dowex 50 (H⁺), filtered and dried under nitrogen. The borate was removed by adding 0.5 mL aliquots of methanol and evaporating to dryness three times. The oxidation products were taken up in 2 M acetic acid and hydrolyzed at 80°C for 2 h. This hydrolysate was dried under a stream of nitrogen. The residue was dissolved in 100 μL of H₂O and analyzed by paper chromatography in 7:5:3 ethanol: 1 M ammonium acetate pH 7.0 solvent system. Radioactive spots were detected with a Molecular Dynamics Quant software model 445 SI phosphor-imager and evaluated with Image Analysis software.

**1,2-Diamino-4,5-methylenedioxybenzene labeling**

The reaction supernatants described in the Polysialyltransferase polymerization assay section were labeled with DMB (1,2-diamino-4,5-methylenedioxybenzene) (Inoue et al. 2001; Inoue and Inoue 2003) with minor modifications described here. DMB forms a fluorescent product with the reducing terminal sialic acid residue of the polymer. After the reaction, supernatants were incubated 48 h, 10 μL of 0.5 M NaOH was added and the reaction was incubated for 16 h at room temperature. The pH was adjusted to 8 with 0.1 M acetic acid. The fluorescence detector was set at 373 nm for excitation and 448 nm for emission according to Hara et al. (1989).

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**Conflict of interest statement**

None declared.

**Abbreviations**

CMP-9-N3-NeuNac, CMP-9-azido-N-acetylenuraminic acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DP, degree of polymerization; FCHASE, 6-(fluorescein-5-carboxamido) hexanoic acid, succinimidyl ester; GD3, neuNac-α2,8-neuNac-α2,3-gal-β1,4-glc-ceramide; GD3-FCHASE, neuNac-α2,8-neuNac-α2,3-gal-β1,4-glc-β-aminophenyl FCHASE; GT3-FCHASE, neuNac-α2,8-neuNac-α2,8-neuNac-α2,3-gal-β1,4-glc-β-aminophenyl FCHASE; HPLC, high-performance liquid chromatography; LB, Luria–Bertani; MW, molecular weight; PST, polysialyltransferase; TLC, thin layer chromatography.

**References**


