

## Characterization and Acceptor Preference of a Soluble Meningococcal Group C Polysialyltransferase<sup>∇†</sup>

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Received 6 August 2010/Accepted 17 January 2011

**Vaccines against *Neisseria meningitidis* group C are based on its  $\alpha$ -2,9-linked polysialic acid capsular polysaccharide. This polysialic acid expressed on the surface of *N. meningitidis* and in the absence of specific antibody serves to evade host defense mechanisms. The polysialyltransferase (PST) that forms the group C polysialic acid (NmC PST) is located in the cytoplasmic membrane. Until recently, detailed characterization of bacterial polysialyltransferases has been hampered by a lack of availability of soluble enzyme preparations. We have constructed chimeras of the group C polysialyltransferase that catalyzes the formation  $\alpha$ -2,9-polysialic acid as a soluble enzyme. We used site-directed mutagenesis to determine the region of the enzyme necessary for synthesis of the  $\alpha$ -2,9 linkage. A chimera of NmB and NmC PSTs containing only amino acids 1 to 107 of the NmB polysialyltransferase catalyzed the synthesis of  $\alpha$ -2,8-polysialic acid. The NmC polysialyltransferase requires an exogenous acceptor for catalytic activity. While it requires a minimum of a disialylated oligosaccharide to catalyze transfer, it can form high-molecular-weight  $\alpha$ -2,9-polysialic acid in a nonprocessive fashion when initiated with an  $\alpha$ -2,8-polysialic acid acceptor. *De novo* synthesis *in vivo* requires an endogenous acceptor. We attempted to reconstitute *de novo* activity of the soluble group C polysialyltransferase with membrane components. We found that an acapsular mutant with a defect in the polysialyltransferase produces outer membrane vesicles containing an acceptor for the  $\alpha$ -2,9-polysialyltransferase. This acceptor is an amphipathic molecule and can be elongated to produce polysialic acid that is reactive with group C-specific antibody.**

*Neisseria meningitidis* groups B and C are the most common causes of meningococcal meningitis in adolescents and adults in Canada, Europe, and the United States. In the United States, 95% to 97% of cases of meningococcal disease are sporadic; however, since 1991, the frequency of localized outbreaks has increased (12, 13). Most of these outbreaks have been caused by serogroup C. Several vaccines based on the meningococcal capsular polysaccharides have been licensed. A tetravalent vaccine consisting of meningococcal groups A, C, Y, and W-135 has also been licensed. Subsequently, a conjugate vaccine of the same serogroup polysaccharide was licensed in the United States (3, 28). In addition there are two meningococcal group C conjugate vaccines licensed in Europe. These meningococcal capsular polysaccharides are polysialic acids. The *N. meningitidis* group B polysaccharide is an  $\alpha$ -2,8-linked polysialic acid, while the group C polysaccharide is an  $\alpha$ -2,9-linked polysialic acid (see Fig. S1 in the supplemental material).

The gene clusters responsible for the synthesis of these polysialic acids have been identified and characterized (5, 7, 10, 20, 24, 27). The glycosyltransferase genes of meningococcal gene clusters have been useful targets for the development of epi-

demiological tools. For instance, PCR assays based on the polysialyltransferase (PST) genes are routinely used for the detection and identification of serogroups (15). The polysialic acids are polymerized by a single polysialyltransferase in the case of each serogroup. The group B polysialyltransferase (NmB PST) is encoded by *synD*, and the group C PST is encoded by *synE*. Several investigators have cloned and begun to characterize the group B polysialyltransferase (9, 34). Both enzymes are membrane associated and are presumed to transfer sialic acid from the donor, CMP-NeuNAc, to the growing chain on the internal face of the cytoplasmic membrane of the bacterium (19, 21, 33).

Much of the work on the biosynthesis of polysialic acid capsules has been done on the *Escherichia coli* K1 and K92 polysialyltransferases (21, 22). Like the meningococcal enzymes, the *E. coli* polysialyltransferases are associated with the cytoplasmic membrane and transfer sialic acid to the nonreducing end of the acceptor chain. Neither *E. coli* nor meningococcus can initiate synthesis *de novo*; they require an oligosialic acid or an endogenous acceptor (9, 17). The endogenous acceptor for these enzymes has yet to be identified. The meningococcal polysialyltransferase has a longer carboxyl-terminal sequence than the *E. coli* enzymes. The bacterial polysialyltransferases do not share motifs or sequence homologies with other sialyltransferases. *E. coli* and meningococcal polysialyltransferases belong to the CAZy glycosyltransferase family GT-38 (6).

Until recently the characterization of the bacterial polysialyltransferases has been limited to studies with membrane

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† Supplemental material for this article may be found at <http://j.b.asm.org/>.

<sup>∇</sup> Published ahead of print on 28 January 2011.

TABLE 1. Plasmid constructs

Plasmid	Gene(s)	Primer	Sequence
pWV234	<i>synE-nusA</i>	CPST LIC for CPST LIC rev	GACGACGACAAGATGTTGCAGAAAATAAGA GAGGAGAAGCCCGGTTTAATTGGTTACAAAGGC
pWV235	<i>synE-SUMO</i>	CPST-SUMO Met for CPST-SUMO rev	ATGTTGCAGAAAATAAGAAAA TTATTGGTTACAAAGGCTATA
pWV241	<i>synE</i>	NmCPST fwd pENTR TOPO NmCPST BamHI rev	CACCATGTTGCAGAAAATAAGAAAAGCT TTTGGATCCTTATTGGTTACAAAGGCTATATTT
pWV237	<i>synD</i>	NmB directional for NmB rev HindIII	CACCATGCTAAAGAAAATAAAAAAAGC AAGCTTTTATCTATCTCTACCAATTCT
pWV238	<i>synD</i>	NmB directional for NmB rev HindIII	CACCATGCTAAAGAAAATAAAAAAAGC AAGCTTTTATCTATCTCTACCAATTCT
pWV245	<i>synD-synE</i>	DSnmB FOR DSnmB REV	GAGGAAAACCTGTATTTTCAGGGCT TAAATGAGTCGTAATATTCTTTTTTTTTTGC
pWV246	<i>synE-Halo tag</i>	NmC PST Flexivector forward NmC PST Flexivector reverse	AGGAGCGATCGCCATGTTGCAGAAAATAAGAAA AACTGTTTAAACTTATTGGTTACAAAGGCTAT
pWV248	<i>synE</i>	NmCPST fwd pENTR TOPO MengC PST rev primer	CACCATGTTGCAGAAAATAAGAAAAGCT TTATTGGTTACAAAGGC
pWV249	<i>synD-synE</i>	B322 primer C322 primer 151 primer C 151 primer D	GCGGGTCTCTTTTTTAGGAGTTATATTATT GCGGGTCTCAAAAAATTCCTATATCCAGC GCGGGTCTCCATTCTACGAAAACCTGTA CCCAGTGCTGCAATGATACCGCGAGA
pWV250	<i>synD-synE</i>	QCL NmC322 sense primer  QCL NmC322 antisense primer	CCTAATAATAACTCTAAAAAATTCCTATATATCCA CGGTGGATATAAGA TCTTATATCCACGCTGGATATATAGGAATTTTTTAGG AGTTATATTATTAGG

fragments of cells harboring the polysialyltransferase genes or *in vivo* experiments (17, 23, 27, 29, 30). Soluble enzyme was not available for structure-function studies due to resistance of the membrane-associated enzymes to extraction in active form with detergents (17). The expression of some soluble membrane proteins has been achieved without detergents by fusion to proteins that promote solubilization. Recently, Freiburger et al. and Willis et al. (9, 34) demonstrated the ability to produce soluble group B polysialyltransferase as a chimera of *synD* and the *malE* or *nusA* gene.

In our study, we constructed several soluble chimeras of the group C polysialyltransferase. The chimeric enzymes were expressed in *E. coli* and purified. The activity of the purified enzymes clearly demonstrated that only a single protein is required for elongation of polysialic acid acceptors.

MATERIALS AND METHODS

**DNA manipulations.** Recombinant DNA techniques were carried out using standard methods and commercially available materials. PCR amplifications were performed using either *Taq* Ready-to-Go beads (GE Healthcare) or a proofreading DNA polymerase, Phusion HF, purchased from New England BioLabs. Transformants were screened by either restriction digestion of plasmid minipreps or directly by PCR. Freshly picked colonies for PCR screening were boiled in diethyl pyrocarbonate water for 5 min and centrifuged. The supernatant was mixed with 1 µl of appropriate primers and Ready-to-Go PCR beads and then amplified in a thermocycler and analyzed on agarose gels.

**Construction of expression plasmids.** Plasmids and primers used for our constructions are described in Table 1.

**pWV234 NusA-PST.** The pWV234 NusA-PST plasmid encodes an amino-terminal NusA fusion with the NmC PST (*synE*). The *synE* gene was amplified from chromosomal DNA isolated from *N. meningitidis* group C strain P2181 with the forward primer CPST LIC FOR and the reverse primer CPST LIC REV and *Taq* Ready-to-Go PCR beads. The amplified fragment was purified and ligated into the expression vector pET44 Ek/LIC containing the *nusA* gene (Novagen) as described in the manufacturer's instructions.

**pWV235 SUMO-PST.** For WV235 SUMO-PST, the *synE* gene was amplified as described above by using plasmid pWV234 as template with forward primer CPST-SUMO Met FOR and reverse primer CPST-SUMO REV. The amplified

fragment was ligated in the pETSUMO TOPO plasmid as recommended by Invitrogen.

**pWV237.** For pWV237, *N. meningitidis* group B *synD* was amplified from chromosomal DNA isolated from strain H355 using primers NmB rev HindIII and NmB directional for. The resulting fragment was ligated into the pET151 TOPO vector from Invitrogen.

**pWV241.** For pWV241, the Gateway system of Invitrogen was used to construct a chimera of the maltose binding protein (MBP) gene, *malE*, and the NmC PST gene, *synE*. The *synE* gene was introduced into an entry vector as follows. The *synE* gene was amplified from pWV234 with the forward primer NmC PST FWD pENTR TOPO, the reverse primer NmC PST BamHI REV, and the thermostable polymerase reagent Phusion HF master mix from Finnzymes. The purified fragment was ligated into the Invitrogen pENTR/TEV/D-TOPO plasmid as recommended by the manufacturer.

**pWN602 *malE* Gateway destination vector.** The plasmid pN-WWW was a gift of Warren Wakarchuk, National Research Council, Canada, and is based on pCWori+ from the same source. pN-WWW was digested with NdeI and HindIII and converted to blunt ends with mung bean nuclease and calf intestine alkaline phosphatase. The Gateway cassette B was inserted into the blunt end site as described by Invitrogen. The resulting plasmid, pWN602, contained the Gateway B cassette at the 3' end of the *malE* gene.

**pWV243 MBP-PST.** For pWV243 MBP-PST, the *synE* (NmC PST) gene in pWV241 was used as the insert gene for the LR clonase (Invitrogen) reaction. pWN602 was used as the destination vector for pWV243. The protein expressed by this plasmid has an MBP at the amino-terminal end of NmC PST separated by a tobacco etch virus (TEV) protease cleavage site.

**pWV246.** For pWV246, the HaloTag fusion plasmid was constructed by inserting the *synE* gene into the expression plasmid pFN22K HaloTag Flexivector (Promega). The *synE* gene was amplified from the plasmid pWV243 with the Phusion Hi-Fidelity enzyme mix (New England BioLabs) using the primers NmC PST Flexivector forward and NmC PST Flexivector reverse.

**pWV239.** For pWV239, the NmB PST gene, *synD*, was cloned into the *malE*-containing Gateway vector pWN602 in a fashion similar to that described for pWV243 above. The *synD* gene was first amplified using primers NmB directional for and NmB rev HindIII and then inserted in pENTR/TEV-TOPO. The resulting plasmid, designated pWV238, was combined with pWN602 in an *in vitro* recombination reaction mixture to yield pWV239. The resulting plasmid expresses a MalE-PST chimera with MalE and the amino terminus of NmCPST separated by a TEV protease cleavage site.

**pWV248.** For pWV248, the NmC PST *synE* gene was amplified with pWV234 as PCR template and NmCPST fwd pENTR TOPO and MengC PST rev as

primers. The resulting NmC PST *synE* was ligated into pET151TOPO as described by the manufacturer.

**Mutagenesis.** The domain swap mutants of NmB and NmC PST were constructed by two methods. The plasmid pWV245 was constructed with the QuikChange II XL kit (Stratagene). The sequence encoding the amino terminus of SynD (nucleotides 1 to 439) was amplified using the primers DSnmB FOR and DSnmB REV with the NmB PST plasmid pWV239 as a template. The resulting fragment was purified and used as a mutagenic primer with the QuikChange II XL kit to mutate the NmC PST expression plasmid pWV243. This procedure requires significant areas of homology in the regions to be swapped. Because of this requirement for homology, the QuikChange method was not suitable for swapping the desired shorter region of *synD*. A modification of the method of Stemmer and Morris (25) was used to construct the other *synD-synE* chimera, although inverse PCR was not performed (4). Briefly, nucleotides 1 to 322 were amplified with a primer that included nucleotides 322 of *synD* in a BsaI cleavage site and a pET151 vector-based primer, including its BsaI site from pWV237. Similarly, nucleotides 322 to 1479 of *synE* were amplified with a primer that included nucleotides 322 of *synE* in a BsaI cleavage site and a *synE* primer that included its BsaI site from pWV248. The fragments were isolated, cut with BsaI, and ligated together. The resulting hybrid fragment was ligated into the BsaI sites of pWV248 to yield plasmid pWV249. Plasmid pWV249 was sequenced and found to have a deletion error in the region of one of the primers used in this construction. The error was corrected using a QuikChange Lightning mutagenesis kit to give pWV250. The sequences of all plasmids were confirmed by sequencing in the CBER Core Facility.

The plasmids pWV245 and pWV250 were transformed into *E. coli* BL21(DE3) cells, and the polysialyltransferase activities of cell lysates were determined as described below.

**Protein analysis.** The protein concentration was determined using the Bio-Rad dye binding reagent. Purified enzyme fractions were analyzed by SDS-PAGE (Invitrogen) and stained with Coomassie blue. HaloTag proteins were labeled as described above, with a tetramethylrhodamine (TAMR)-HaloTag, and analyzed by SDS-PAGE with fluorescence detection.

**Polysialyltransferase expression and purification.** The NusA polysialyltransferase chimera was expressed by transformation of plasmid pWV234 into BL21(DE3) Star and preparation of an overnight inoculum in 25 ml of LB containing 200 µg/ml ampicillin. The culture in LB broth (1.5 liters) containing 50 µg/ml ampicillin was inoculated and shaken at 30°C until the absorbance at 600 nm reached 1.2. The culture was induced by addition of 360 mg isopropyl-β-D-thiogalactopyranoside (IPTG) and shaking at 30°C for an additional 2 h 45 min. The cells were harvested by centrifugation at 10,000 × g for 15 min, at 4 to 10°C, and stored at -80°C. Frozen cells were thawed and suspended in 10 ml 50 mM Tris, 10 mM MgCl<sub>2</sub> in which was dissolved one EDTA-free protease inhibitor tablet (pH 7.5; Roche). The cell suspension was lysed in a French pressure cell and centrifuged for 12 min at 10,000 × g, at 4 to 10°C. Membranes were separated from the cytosolic fraction by ultracentrifugation for 1 h at 114,000 × g at 4°C. The membrane fraction was resuspended in 1.5 ml of cryo buffer (31). The supernatant fraction was adjusted to 10% glycerol and stored at -80°C.

Polysialyltransferase constructs with a His tag epitope were purified from the supernatant by tumbling overnight at 2 to 8°C with 4 ml of Ni-nitrilotriacetic acid-agarose resin (Qiagen) in 50 mM Tris, 10 mM imidazole, pH 7.5. The suspension was poured into a column and washed at 0.5 ml/min with 50 mM Tris, 10 mM imidazole, 200 mM NaCl, pH 7.5. The protein was eluted with the same buffer containing 60 mM imidazole and then 250 mM imidazole, collecting 3-ml fractions. Fractions were assayed for activity using the paper chromatography assay described below. Peak activity fractions were combined and concentrated using a Centriplus YM10 apparatus from 4 ml to 1 ml. The concentrated enzymes was adjusted to 10% glycerol and stored at -80°C.

The SUMO-polysialyltransferase chimeric enzyme was purified from cells harboring the plasmid pWV235 by a similar procedure, except the culture was grown in LB with kanamycin.

The MaIE-NmC polysialyltransferase was purified as follows. Plasmid pWV243 was transformed into AD202 cells, and the fresh transformants from the entire plate were suspended in a few milliliters of medium and used to inoculate 1.5 liters of rich broth (consisting of 15 g Bacto tryptone, 7.5 g yeast extract, 7.5 g NaCl) plus 3 g glucose and 100 µg/ml ampicillin (pH 7.2). Cells were shaken at 30°C for 4 h and then induced for 3 h with 400 mg IPTG. A cytosolic fraction was prepared as described above, and the enzyme content was purified on a 12-ml amylose resin (New England BioLabs) column. The amylose column was washed with 50 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.4, and the protein was eluted with 10 mM maltose in the same buffer.

**Preparation of fluorescent-tagged polysialyltransferase.** HaloTag-NmC PST was prepared by transforming pWV246 in BL21(DE3) STAR cells. The fresh

transformants were used to inoculate 2 × 1.5 liters of LB-kanamycin and grown to an *A*<sub>600</sub> of 0.7, then induced with 1 mM IPTG for 3 h. The cells were harvested and frozen in 25 ml 50 mM Tris, 25 mM MgCl<sub>2</sub> (pH 8) overnight. The cell suspension was lysed in a French pressure cell, and cellular debris was removed by centrifugation at 10,000 × g for 15 min.

The lysate (25 ml) was labeled with 250 µl of TAMR-HaloTag substrate (10 to 20 µg/ml) as described by Promega. The membranes were separated from the soluble fraction by ultracentrifugation at 114,000 × g. The membrane pellet was resuspended in 4 ml cryoprotective buffer.

**Gel filtration of HaloTag-NmC PST.** The soluble fraction described above was adjusted to 25% saturated ammonium sulfate and stirred at 4°C for 1 h. The pellet was collected by centrifuging at 15,000 rpm for 30 min and dissolved in 3 ml of 50 mM Tris, 25 mM MgCl<sub>2</sub> (pH 8.0). Most of the enzyme activity in the soluble fraction was precipitated at 25% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation. The enzyme fraction was analyzed on a Superose 12 fast-protein liquid chromatography (FPLC) column (GE) on a Dionex Summit high-performance liquid chromatography (HPLC) equipped with a Hitachi model L-7485 fluorescence detector. TAMR-labeled fractions were detected at an excitation wavelength of 555 nm and emission at 585 nm. Peak fractions were concentrated on Nanosep micro-concentrators and assayed for polysialyltransferase activity as described previously (17).

**Assay for polysialyltransferase.** Polysialyltransferase activity was measured using a paper chromatography assay described previously (17).

The linkage of the product of the chimera plasmids pWV245 and pWV250 was determined as follows. In a typical assay, the membrane fraction was incubated in a solution containing 22 mM Tris, 11 mM MgCl<sub>2</sub>, pH 8, 10 µg polysaccharide acceptor (NmC polysaccharide, colominic acid, or K92 polysaccharide) in 110 µl and 3 µl 100-µCi/ml <sup>14</sup>C-labeled CMP-sialic acid (American Radiolabeled Chemicals). After a 1-h incubation, α-2,8-endoneuraminidase was added, and incubation at 37°C continued for 3 h, prior to analysis by paper chromatography. Control reaction mixtures were incubated for 3 h without endoneuraminidase.

**NmC PST competition assay with unlabeled acceptors.** Lactosyl-boron dipyrromethene label (BODIPY) was sialylated with the bifunctional sialyltransferase CST II in a large-scale reaction volume (500 µl), and oligosialylated products were separated by anion exchange HPLC as described before (16). Product peaks were collected and desalted using Sep Pak chromatography (Waters). The extent of sialylation was confirmed by matrix-assisted laser desorption ionization-mass spectrometry (data not shown). Trisialylated lactosyl-BODIPY (2.5 µM) was incubated with NmC PST (30 µg/ml) in the presence of CMP-NeuNAc (5 mM) and MnCl (40 mM) in 200 mM sodium cacodylate buffer (pH 8.0) at 37°C for 40 min. For competition experiments, the reaction was run in the presence of sialic acid trimer, hexamer, or GD3 (Calbiochem) at a concentration of 5 µM. All reactions were quenched by the addition of ethanol to 25%. Products were dried by using a Speedvac, brought up to 50 µl with H<sub>2</sub>O, and subjected to HPLC analysis as previously described (18).

**Preparation of outer membrane vesicles.** Outer membrane vesicles (OMV) were prepared from cultures of mutant *N. meningitidis* group B strains according to the procedure described by Frasch and Pepller (8). Cultures were grown overnight on tryptic soy broth supplemented with yeast extract as described by Arakere et al. (2). *N. meningitidis* strain M7 lacking the ability to synthesize sialic acid and a *synD* mutant lacking an active polysialyltransferase were obtained from David Stephens, Emory University (14).

**Detection of polysialic acid formation by ELISA.** NmC PST (13 µl) was incubated with 10 µl OMV and CMP-NeuNAc (33, 330, 1,000, or 2,000 µM) in a total of 30 µl as described previously (17) for 1 h at 37°C. The assay mixture was then diluted to 400 µl with enzyme-linked immunosorbent assay (ELISA) coating buffer (2), which was then used to coat Immulon 1B plates (100 µl/well) at room temperature overnight. The plates were washed and then incubated with mouse monoclonal IgG antibody Mcl2075 (against OAc<sup>+</sup>) or Mcl2016 (against OAc<sup>-</sup>) group C polysaccharide at 1:400 dilution (kindly donated by Marjorie Shapiro, FDA) (11). Plates were developed with goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) diluted 1:2,000.

## RESULTS AND DISCUSSION

**Distribution and purification of PST activity.** The polysialyltransferases of *Neisseria meningitidis* groups B and C are encoded by *synD* and *synE*, respectively. Experiments by Freiburger et al. (9) demonstrated that a soluble and active group B α-2,8-polysialyltransferase could be expressed by some chimeras of *synD*. We prepared several chimeras of *synE* that were capable

TABLE 2. Distribution of polysialyltransferase activity

Plasmid	Expression chimera	Activity (cpm/min/mg of protein) in:	
		Membranes	Supernatant
pWV234	NusA-NmC	1,800	1,868
pWV243	MalE-NmC	2,277	2,669
pWV235	SUMO-NmC	2,453	91
pWV245	HaloTag-NmC	4,148	5,671

of expressing soluble *N. meningitidis* group C  $\alpha$ -2,9-polysialyltransferase. As shown in Table 2, most of the chimeras that we constructed expressed group C  $\alpha$ -2,9-polysialyltransferase activity equally in the membrane and supernatant fractions of cell lysates. The expression levels of the chimeric protein in the membrane and supernatant fractions were confirmed by Western blot analysis of the MalE-PST chimera (Fig. 1). Membrane and cytosolic fractions of pWV243 lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and immunostained with anti-MalE antibody. Bands with a mass of 84 kDa were observed in both fractions. Although several chimeras of the NmC PST yielded a significant amount of enzyme activity in the soluble fraction, we obtained a high yield of purified enzyme with the maltose binding protein chimera. The maltose binding protein chimera was stable when stored at  $-80^{\circ}\text{C}$  in 10% glycerol and was therefore used for most of the characterization experiments described below.

**Acceptor domain.** The NmC and NmB polysialyltransferases synthesize different polysialic acid homopolymers and share 75% sequence homology and 64% identity. The product of the NmC enzyme is  $\alpha$ -2,9-polysialic acid, while that of NmB is the more common  $\alpha$ -2,8 polymer. *E. coli* and *N. meningitidis* polysialyltransferases are only about 40% homologous, yet the *E. coli* K1 and *N. meningitidis* group B polysialyltransferases synthesize identical polysialic acids. This leads one to wonder which amino acid differences in the NmC and NmB polysialyltransferases are associated with the linkage differences in the polysaccharide products. Steenbergen et al. (22) showed that the region of the *E. coli* K1 and K92 enzymes responsible for the synthesis of different polysialic acid linkages is contained within the amino acid sequence of residues 1 to 100 of the *E. coli* polysialyltransferases. Similarly, we reasoned that

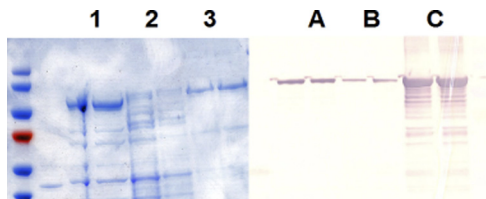


FIG. 1. Distribution of the MalE-NmC PST chimera, after PAGE of purified MalE-NmC PST. The ultracentrifugation pellet (lane 1), supernatant of a cell lysate of AD202(pWV243) (lane 2), and the major protein fractions eluted from the amylose column (lane 3) were loaded onto a 4-to-20% polyacrylamide SDS-PAGE gel. For Western blot analysis another gel was loaded with membrane fraction (lane A), the ultracentrifuge supernatant fraction of the BL21(pWV243) lysate (lane B), and the amylose-purified MalE-NmC PST (lane C). The gel was transferred to nitrocellulose, and the immunoblot was developed with anti-MalE antibody.

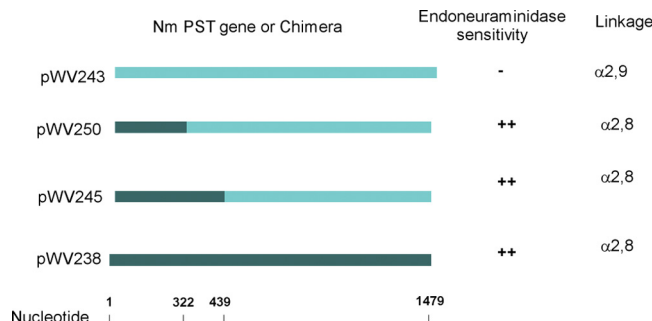


FIG. 2. Domain swap of NmB PST and NmC PST. Chimeras were constructed with nucleotides 1 to 439 of *synD*, 440 to 1479 of *synE* (pWV250), 1 to 322 of *synD*, and 323 to 1479 of *synE* (pWV245) as described in Materials and Methods. The chimeric enzymes were expressed and assayed, and the linkages of their polysialic acid products were determined by the sensitivity of the enzymatic reaction products to the  $\alpha$ -2,8-NeuNAc-specific endoneuraminidase as described in Materials and Methods.

we could localize the amino acids responsible for the link differences in the polysialic acid products by domain swapping of the group B and C polysialyltransferases. We used site-directed mutagenesis to determine the region of the *Neisseria* polysialyltransferases required to synthesize  $\alpha$ -2,8 or  $\alpha$ -2,9 linkages. Chimeras were constructed with nucleotides 1 to 439 of *synD*, nucleotides 440 to 1479 of *synE*, nucleotides 1 to 322 of *synD*, and nucleotides 323 to 1479 of *synE* (Fig. 2). The chimeric enzymes were expressed, and the linkage of their polysialic acid products was determined. The products of the reaction with  $\text{CMP-}[^{14}\text{C}]\text{NeuNAc}$  were sensitive to the  $\alpha$ -2,8-specific endoneuraminidase (1, 22), suggesting that the enzymes now had  $\alpha$ -2,8 linkage specificity (Fig. 2). Both *synE* and the *synD-synE* chimera encoded enzymes that utilized  $\alpha$ -2,8-polysialic acid,  $\alpha$ -2,9-polysialic acid, and  $\alpha$ -2,8  $\alpha$ -2,9 polysialic acids as acceptors. Both chimeras synthesized  $\alpha$ -2,8-polysialic acid, suggesting that like the *E. coli* enzyme the amino acid sequence sufficient to determine the linkage of the product is contained within residues 1 to 107.

**Active molecular weight.** It has been proposed that bacterial glycosyltransferases responsible for synthesis of some capsular polysaccharides function as members of a complex (31, 33). Thus, we investigated the active molecular weight of NmC PST. The HaloTag-PST chimera can be fluorescently labeled with selective reagents (16), and it therefore provided a means of detecting the molecular species of polysialyltransferase present in a cell lysate without extensive purification. We prepared a crude ammonium sulfate fraction of fluorescently labeled HaloTag-PST for analysis by HPLC size exclusion chromatography. We constructed a plasmid that expressed the group C polysialyltransferase fused to a HaloTag protein, which reacts covalently with specific chloroalkane substrates. The chimeric polysialyltransferase isolated from a capsule-free *E. coli* background was labeled with TAMR-HaloTag substrate prior to ammonium sulfate fractionation to determine which molecular sizes were active in the native state. Two major fluorescent species were observed by HPLC, a high-molecular-weight (MW) aggregate and a monomer. Both species were active in the PST assay when meningococcal group C polysaccharide was used as the acceptor. Most of the activity was observed in

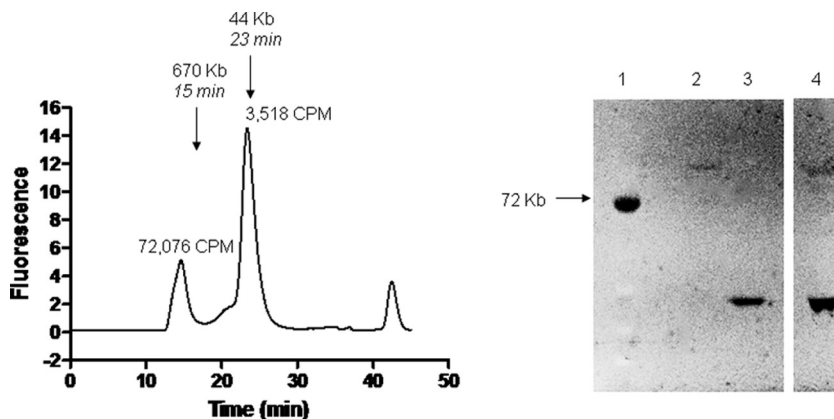


FIG. 3. Native molecular size of the HaloTag-NmC PST chimera. A crude 25% saturation ammonium sulfate fraction of cell supernatant containing HaloTag-NmC polysialyltransferase was labeled as described in Materials and Methods. (A) The labeled lysate was applied to a Superose 12 FPLC column, and peak fractions were assayed for activity as described in Materials and Methods. (B) The labeled lysate (lane 4) was also applied to an SDS-PAGE gel, and labeled proteins (lanes 2 and 3) were detected under UV light.

the high-MW peak. Gel electrophoresis of the peak fraction suggested that the high-molecular-weight fraction contained mostly the PST chimera (Fig. 3), while the low-molecular-weight fraction contained mostly HaloTag. Little or no fluorescent chimera was detected by SDS-PAGE gels in the low-molecular-weight fraction. These results suggest that the meningococcal group C polysialyltransferase is active as a monomer but has a tendency to form high-MW aggregates. This result is consistent with the enzyme being a membrane-associated protein and having exposed hydrophobic sites.

**Metal ion requirement, pH optimum, and kinetics.** The NmC PST-MalE chimera exhibits a broad pH dependence of activity, with a maximum activity obtained at pH 8.0. Polysialyltransferase activity is stimulated by the divalent cations  $Mg^{2+}$  and  $Mn^{2+}$ ; however, the NmC PST does not require a metal ion cofactor. Enzyme fractions were active after treatment with the metal chelators EDTA and dipicolinic acid (26). The  $K_m$  for CMP-NeuNAc was determined using a radiolabel assay with NmC polysaccharide. The  $K_m$  for CMP-NeuNAc was 432  $\mu M$  for NmC PST, which is very close to the value reported for the MalE-NmB PST chimera, 420  $\mu M$  (9).

**Acceptor activity of polysaccharides.** The NmC PST can extend polysialic acid and oligosialic acid acceptors. We tested the ability of the NmC PST to use *E. coli* K1 and K92 polysaccharides as well as the meningococcal group C polysialic acid as acceptors. All polysaccharides supported sialic acid transfer in the radiolabel assay. We previously showed that the *E. coli* and meningococcal group B polysialyltransferases are able to extend the fluorescent disialylated oligosaccharide to form high-molecular-weight polysialylated products in a non-processive fashion (9). Figure 4 shows the time-dependent extension of GD3-FCHASE to form high-molecular-weight polymer. The product was confirmed to be  $\alpha$ -2,9 linked by the resistance to the  $\alpha$ -2,8-specific endoneuraminidase. The high-MW fluorescent product was isolated and analyzed by HPLC size exclusion chromatography. The results of this experiment suggested a species with a mass greater than the 670-kDa marker.

The fact that the disialylated GD3 analogue is a substrate is consistent with the enzyme using  $\alpha$ -2,8-NeuNAc K1 polysaccharide as an acceptor. This result implies that although NmC

PST requires at least a disialylated acceptor, it does not require 2,9-linked acceptors to form 2,9-polysialic acid. In Fig. 5 the disialylated ganglioside GD3 and a hexasaccharide of K92 possessing  $\alpha$ -2,9-linked disialic acid at the nonreducing end are compared. In the radioactivity assay, the ganglioside GD3 was a much better acceptor than the K92 oligosaccharide. Although the K92 oligosaccharide has a 2,9-disialic acid at the nonreducing end, at least 10-fold less sialic acid was incorporated when it was used as an acceptor. This suggests that at least for short oligosaccharides the NmC PST prefers a hydrophobic region in addition to the oligosialic acid. The preference for hydrophobic acceptors was tested further in a competition experiment. In this experiment we measured the ability of unlabeled acceptor to compete with the fluorescent acceptor trisialyllactosyl-BODIPY by HPLC (18). As can be seen in Fig. 6, the disialyl ganglioside GD3 was a better inhibitor of elongation of the fluorescent substrate than the trisialyl oligosaccharide. The observation that the elongation reaction can be inhibited by competition with other acceptor substrates is consistent with the notion that the *in vitro* elongation reaction is not processive.

**NmC PST can transfer to a hydrophobic acceptor in outer membrane vesicles.** We prepared outer membrane vesicles from a strain of *N. meningitidis* group B lacking sialyltrans-

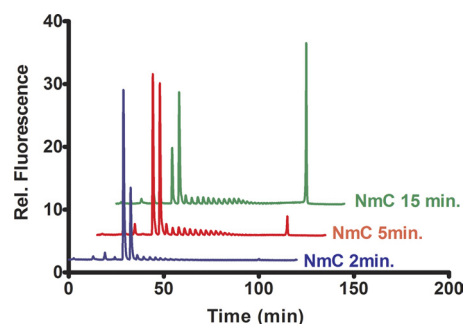


FIG. 4. Time course of GD3-FCHASE elongation. Purified MalE-NmC PST was incubated with the fluorescent acceptor GD3-FCHASE and analyzed by ion exchange HPLC as previously described (32).

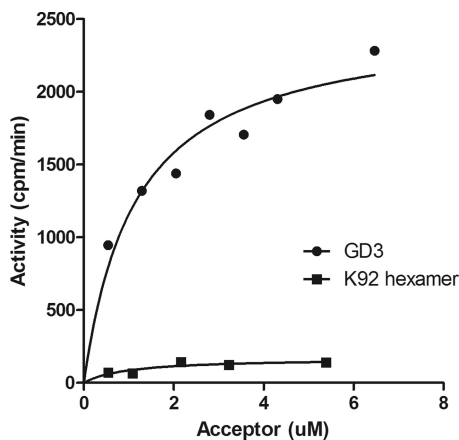


FIG. 5. Comparison of acceptor activity of GD3 and sialic acid hexasaccharide. The ganglioside GD3 and a hexasaccharide isolated from the K92 polysaccharide were incubated as acceptors with purified MalE-NmC PST and CMP-[<sup>14</sup>C]sialic acid in an assay described in Materials and Methods. The hexasaccharide was prepared by digestion of K92 polysaccharides and purification by ion exchange and gel filtration as described previously (17, 32).

ferase activity and therefore unable to synthesize a polysialic acid. We tested these vesicles for their level of an endogenous polysialyltransferase acceptor. We confirmed that these preparations were indeed outer membranes, based on their SDS-gel electrophoresis profiles and sucrose density gradient fractionation. The protein profile detected by SDS-gel electrophoresis was characteristic of *N. meningitidis* outer membranes. The OMV fraction was also loaded onto a sucrose density gradient and assayed for the cytoplasmic membrane marker enzyme lactate dehydrogenase. No lactate dehydrogenase activity was detected, and thus no detectable cytoplasmic membranes were present.

These vesicles were tested for the presence of an acceptor by addition of a polysialyltransferase assay mixture containing the PST-MalE chimera and, as the donor substrate, radiolabeled CMP-sialic acid. The acceptor activity observed in these assays was similar to that expected for the addition of homologous meningococcal polysaccharides (Table 3). To demonstrate that a polysaccharide was being formed on these vesicles, the ELISA plates were coated with these reaction mixtures and

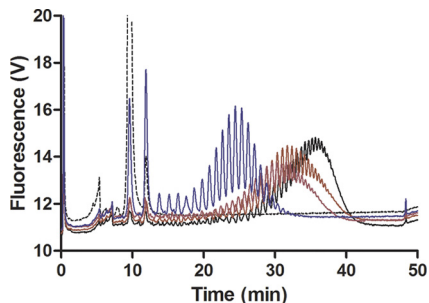


FIG. 6. Competition of the PST elongation reaction with unlabeled acceptors. Trisialylated lactosyl-BODIPY (2.5 μM; dotted trace) was incubated with NmC PST (30 μg/ml) in the absence (black trace) or presence of 5 μM α-2,8-sialic acid trimer (maroon trace), α-2,8-hexamer (purple trace), or GD3 (blue trace).

TABLE 3. Acceptor activity of OMV (*synD*) and phenol-extracted OMV

Acceptor	Polysialyltransferase	Acceptor activity (cpm) <sup>a</sup>
NmC PS	NmC PST	36,460
NmC PS	Boiled PST	165
OMV ( <i>synD</i> )	NmC PST	64,320
OMV (phenol extract)		20,884
C <sub>18</sub> elution solvent		
Aqueous		920
10% CH <sub>3</sub> CN		18,282
30% CH <sub>3</sub> CN		19,092
50% CH <sub>3</sub> CN		1,451

<sup>a</sup> Acceptors were incubated with the polysialyltransferase, and the formation of <sup>14</sup>C-labeled polysialic acid was assayed as described in Materials and Methods.

assayed for the presence of α-2,9-polysialic acid with meningococcal group C-specific antibody (11). As is seen in Fig. 7, the reaction product was reactive with monoclonal antibodies specific for the de-O-acetylated 2,9-polysialic acid (11) in a CMP-NeuNAc concentration-dependent manner.

This acceptor could be extracted from the membrane vesicles by using a buffer phenol-water method. This acceptor is active with *N. meningitidis* B and *E. coli* K1 and K92 polysialyltransferases (data not shown). That this acceptor is hydrophobic is demonstrated by its adsorption to C<sub>18</sub> reverse-phase resin. The acceptor activity contained in the aqueous phase of the phenol extraction was retained on the C<sub>18</sub> resin and could be eluted with 30% acetonitrile (Table 3). The fraction eluted from the C<sub>18</sub> resin did not stain in silver-stained SDS-PAGE gels and therefore probably was not lipopolysaccharide.

In summary, the results described above show that the NmC polysialyltransferase can be expressed and purified as a soluble active enzyme. The soluble enzyme exists in its active form primarily as a high-molecular-weight aggregate. Like the *E. coli* polysialyltransferases, the linkage of the polysialic acid product is determined by the amino terminus of the enzyme. This would suggest that at least part of the acceptor site resides in the amino terminus of the enzyme. While the structure of

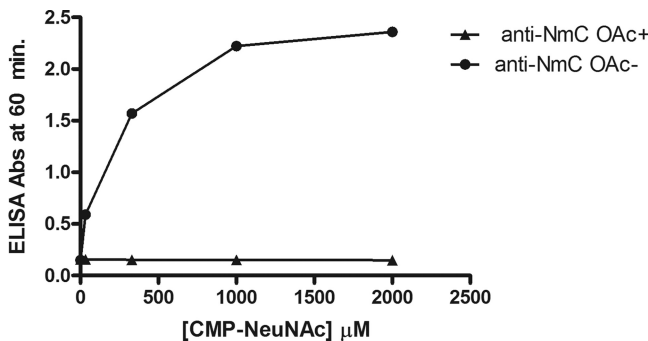


FIG. 7. CMP-NeuNAc dependence of outer membrane acceptor elongation. A reaction mixture containing MalE-NmC PST, CMP-sialic acid, and OMV as described in Materials and Methods was applied to ELISA plates. The presence of meningococcal group C polysaccharide was detected with monoclonal antibodies to O-acetylated (closed triangles) and de-O-acetylated (closed circles) group C polysaccharides as described in Materials and Methods.

the natural acceptor remains unknown, we have learned from these experiments that it probably contains a hydrophobic moiety. This is inferred from the acceptor preference of the NmC polysialyltransferase and from the nature of the acceptor fractions extracted from *Neisseria* membranes.

#### ACKNOWLEDGMENTS

We thank the following investigators for the generous donation of strains and plasmids, Warren Warkachuk, National Research Council, Canada; Eric Vimr, University of Illinois, Urbana, IL; David Stephens, Emory University, Atlanta, GA. We also thank Eric Vimr for helpful discussions.

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