Cloning of the Lipooligosaccharide α-2,3-Sialyltransferase from the Bacterial Pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*

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The genes encoding the α-2,3-sialyltransferases involved in lipooligosaccharide biosynthesis from *Neisseria meningitidis* and *Neisseria gonorrhoeae* have been cloned and expressed in *Escherichia coli*. A high sensitivity enzyme assay using a synthetic fluorescent glyco-syltransferase acceptor and capillary electrophoresis was used to screen a genomic library of *N. meningitidis* MC58 L3 in a “divide and conquer” strategy. The gene, denoted *lst*, was found on a 2.0-kilobase fragment of DNA, and its sequence was determined and then used to design probes to amplify and subsequently clone the corresponding *lst* genes from *N. meningitidis* 406Y L3, *N. meningitidis* M982B L7, and *N. gonorrhoeae* F62. Functional sialyltransferase was produced from the genes derived from both L3 *N. meningitidis* strains and the *N. gonorrhoeae* F62. However, the *N. meningitidis* M982B L7 gene contained a frameshift mutation that renders it inactive. The expression of the *lst* gene was easily detected using the enzyme assay, and the protein expression could be detected when an immunodetection tag was added to the COOH-terminal end of the protein. Using the synthetic acceptor N-acetyllactosamineaminophenyl-(6-(5-(fluorescein-carboxamido)-hexanoic acid amide), the α-2,3 specificity of the enzyme was confirmed by NMR examination of the reaction product. The enzyme could also use synthetic acceptors with lactose or galactose as the saccharide portion. This study is the first example of the cloning, expression, and examination of α-2,3-sialyltransferase activity from a bacterial source.

Mammalian oligosaccharides containing terminal N-acetyl-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U60660, U60661, U60663, and U60664.

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1 The abbreviations used are: Neu5Ac, N-acetylneuraminic acid; LOS, lipooligosaccharide; CE, capillary electrophoresis; FCHASE, 6-(5-fluorescein-carboxamido)-hexanoic acid succimidyl ester; FCHASE-LacNAc, aminophenyl-N-acetyllactosamine-6-(5-(fluorescein-carbox-amido)-hexanoic acid amide); FCHASE-Lac, aminophenyl-lactose-6-(5-fluorescein-carboxamido)-hexanoic acid amide; PCR, polymerase chain reaction; kb, kilobase; MES, 2-(N-morpholino)ethanesulfonic acid pfu, plaque-forming unit; PAGE, polyacrylamide gel electrophoresis; NOE, nuclear Overhauser effect.
Here we describe the first cloning and characterization of a CMP-Neu5Ac:β-galactoside α-2,3-sialyltransferase from the pathogens N. meningitidis and N. gonorrhoeae achieved by the use of a highly sensitive screening procedure based on the expression of enzyme activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—The following N. meningitidis strains were used in this study: immunotype L3 MC58 (NRCC 4728); immunotype L4 406Y (NRCC 4030); and immunotype L7 M982B (NRCC 4725). DNA from N. gonorrhoeae F62 (ATCC 33084) was a kind gift from Dr. Wendy Johnson (Health Canada, Ottawa, ON, Canada).

**Basic Recombinant DNA Methods**—Plasmid DNA isolation, restriction, and DNA fragments for cloning, ligation, and transformations, and DNA sequencing were performed as recommended by the enzyme supplier or the manufacturer of the kit used for the particular procedure. PCR was performed with Pwo polymerase as described by the manufacturer (Boehringer Mannheim). Restriction and DNA modification enzymes were purchased from New England Biolabs Ltd. (Mississauga, ON, Canada). Qiagen columns were used for PCR purification. DNA sequencing was performed with an Applied Biosystems (Montreal, Canada) model 370A automated DNA sequencer using the manufacturer’s cycle sequencing kit. DNA sequence analysis and protein alignments were performed with the Genetics Computer Group suite of programs (Madison, WI).

**Cloning and Sequencing of the Sialyltransferase from N. meningitidis**—A genomic library was prepared using 3–5-kb fragments from a HaeIII partial digest of the chromosomal DNA of N. meningitidis MC58 into ZAPII (Stratagene, La Jolla, CA) as the vector (8). The ZAPII library was plated at low density, and 3600-well isolated plaques were picked in pools of 100. Phage suspensions were made as described previously (12). Reactions for the enzyme assay were performed with 0.2 μl of each phage isolate at a dilution of 1:100, and the cells were then assayed for sialyltransferase activity from E. coli carrying pNST plasmids as measured in toluene-treated cells or with the enzyme was verified by NMR of its product to be a β-galactoside α-2,3-sialyltransferase. By NMR, the complete 1H assignment of the compounds was performed. It was found that the 1H chemical shifts (Table I) were similar to those of reported structures containing α-NeuAc-(2-3)-Gal. Also an NOE across the glycosidic bond Hα,α-sialic acid to H3-Gal confirmed that the β-galactoside α-NeuAc-(2-3)-Gal linkage was present in the product (Fig. 1).

**RESULTS**

**Detection and Characterization of α-2,3-Sialyltransferase Activity from N. meningitidis**—The initial part of this work was performed with the N. meningitidis strain 406Y L3, which possesses an LOS identical to that of strain MC58 but has a different capsular type. Both of these strains elaborate the L3 immunotype LOS, which consists of a lacto-N-neotetraose branch with an α-2,3-sialic acid on the terminal galactosyl residue (13). Both of these strains produced easily detectable levels of α-2,3-sialyltransferase when using as little as a single colony (106 cells) with the CE-based assay. Crude extract from N. meningitidis 406Y L3 was used to prepare material for determination of the linkage of the siaoside being synthesized and the enzyme was verified by NMR of its product to be a β-galactoside α-2,3-sialyltransferase. By NMR, the complete 1H assignment of the compounds was performed. It was found that the 1H chemical shifts (Table I) were similar to those of reported structures containing α-NeuAc-(2-3)-Gal (13). Also an NOE across the glycosidic bond Hα,α-sialic acid to H3-Gal confirmed that the β-galactoside α-NeuAc-(2-3)-Gal linkage was present in the product (Fig. 1).
of five when we infected a 1.5-ml isopropyl-1-thio-

mation of hexanoyl amide (NHCO(CH$_2$)$_4$-

membrane fraction (86% in the cell membrane pellet after

same conditions were also optimal for the enzyme from crude

up to 0.2%. Using this method no activity could be detected in

testedinhibitedtheenzyme,withtheexceptionofTritonX-100

required for activity, and in fact many common detergents

required the addition of CMP-Neu5Ac, and it migrated the

Formation of the product peak in the electropherogram

responds to 20 attomoles (2$^{-15}$mol). Formation of the product peak in the electropherogram corre-

TABLE I

NMR chemical shifts for α-D-Neu5Ac-(2–3)β-D-Galp-(1–4)β-D-

GlcNac-aminophenyl-(6–5)(fluorescein-carboxamido)-hexanoic

acid amide)

| Sugar       | Position | H   | C   |
|-------------|----------|-----|-----|
| GlcNac      | 1        | 4.87| 100.2|
|             | 2        | 3.89| 55.6 |
|             | 3        | 3.71| 73.0 |
|             | 4        | 3.67| 72.3 |
|             | 5        | 3.49| 75.6 |
|             | 6        | 3.88| 60.7 |
|             | 6'       | 3.71|     |
| Gal         | 1        | 4.52| 103.4|
|             | 2        | 3.59| 70.2 |
|             | 3        | 4.12| 76.5 |
|             | 4        | 3.97| 68.3 |
|             | 5        | 3.73| 76.1 |
|             | 6        | 3.77| 61.9 |
|             | 6'       | 3.73|     |
| Neu5Ac      | 3$_{ax}$ | 1.82| 40.6 |
|             | 3$_{ax}$ | 2.77|     |
|             | 4        | 3.69| 69.4 |
|             | 5        | 3.85| 52.4 |
|             | 6        | 3.64| 73.5 |
|             | 7        | 3.56| 69.0 |
|             | 8        | 3.9  | 72.6 |
|             | 9        | 3.88| 63.5 |
|             | 9'       | 3.71|     |
| NAc         | 2.03     | 22.8 |

because it was active in the presence of 5 mM EDTA. These

same conditions were also optimal for the enzyme from crude

extracts of MC58, 406Y, and for the recombinant enzymes

from MC58. The natural enzyme was mostly associated with the cell

membrane fraction (86% in the cell membrane pellet after

centrifugation at 100,000 x g). However, no detergent was

required for activity, and in fact many common detergents

tested inhibited the enzyme, with the exception of Triton X-100

up to 0.2%. Using this method no activity could be detected in

M982B L7 cells.

Cloning and Sequencing of the Sialyltransferase Gene from

N. meningitidis MC58—Using the CE laser-induced fluores-

cence assay, we observed sialyltransferase activity one time out of five when we infected a 1.5-ml isopropyl-1-thio-

galactopyranoside-induced E. coli XL1-Blue culture with 1000 pfu

from the N. meningitidis MC58 genomic library in λZAPII (Fig.

2). Formation of the product peak in the electropherogram

required the addition of CMP-Neu5Ac, and it migrated the

same as the sialidase-sensitive product peak formed by the

natural enzyme. The peak in the CE electropherogram corre-

sponds to 20 attomoles (2 x 10$^{-15}$ mol) of product. Single clones expressing the sialyltransferase were obtained by a "di-

vide and conquer" strategy sequentially screening pools of 100

pfu from the λZAPII library of MC58, pools of 5 pfu derived

from the first positive pool, and finally individual plaques

plated at low density. The initial screening yielded two positive

pools of 100 pfu out of 36. From one of these pools we screened

60 pools of 5 pfu and obtained three positive pools. From the

positive pools of 5 pfu we obtained many individual positive

clones and the pBluescript SK$^-$ phagemids excised from them

were found to carry a 2.0-kb insert.

The 2.0-kb insert was sequenced on both strands (GenBank$^\text{®}$

accession number U60660) and a BLASTX search was perfor-

med in GenBank$^\text{®}$ in order to identify any homology with

previously sequenced genes. This analysis revealed two partial

open reading frames (nucleotides 1–140 and 1853–2039), lo-

cated at the opposite ends of the 2.0-kb insert, that were clearly

homologous with various bacterial isocitrate dehydrogenases

(60–85% identity) and various bacterial cytochrome c proteins

(45–63% identity), respectively. A third open reading frame

(nucleotides 573–1685) was designated lst (lipooligosaccharide

gialyltransferase) and revealed significant homology to a Hae-

mophilus influenzae gene designated lsg-ORF2 (GenBank$^\text{®}$

accession number M94855). Pair-wise alignment between the

translation products of lst and lsg-ORF2 indicated that their amino acid sequences share 29.3% identity and 58.3%

similarity (Fig. 3).

The lst gene has two potential start codons, and the second of

these is more likely to be used because the amino acid sequence

immediately following this start codon appears to be a non-

FIG. 1. $^1$H NMR spectrum of sialylated FCHASE-aminophenyl-

N-acetyllactosamine. $^1$H refers to H1 of GlcNac; 1b, 3b, and 4b denote

the H1, H3, and H4 resonances of Gal; and 3eq, 3ax, 4c, and 5c repre-
sent Neu5Ac signals. The NOE spectrum (bottom panel) taken from the
two-dimensional NOE spectrum shows the NOE from H3$_{ax}$ of sialic acid

to H3 of Gal due to the presence of the Neu5Ac(2–3)Gal linkage in the

final product.

FIG. 2. Detection of activity of the recombinant N. meningitidis

α2,3-sialyltransferase. Two electrophorograms are superim-

posed here to illustrate the level of sialyltransferase activity found in

a 1.5-ml E. coli culture infected with 1000 pfu from the genomic DNA

bank of N. meningitidis MC58 in λZAPII. The thin line is from a run

where the reaction contained no CMP-Neu5Ac donor, and the thick

line is from a run containing the CMP-Neu5Ac donor. The peak at 6.6

min was shown to comigrate with FCHASE-α2,3-sialyl-N-acetyl-

lactosamine. The detector response is expressed in relative fluorescence

units (RFU).
cleavable leader sequence (14), and a potentially very good ribosome binding site (AGGGA) occurs just upstream.

Comparison of Sialyltransferase Genes from Different N. meningitidis Strains and N. gonorrhoeae—Isolation of the genes from N. meningitidis 406Y L3 (GenBank® accession number U60661), M982B L7 (GenBank® accession number U60663), and N. gonorrhoeae F62 (GenBank® accession number U60664) was accomplished with PCR primers based on the gene from MC58 L3 (GenBank® accession U60660). We found 12 base differences, which results in five amino acid differences between the two genes from the L3 immunotype strains (Fig. 4), 19 differences in the gene from M982B L7 compared with that of 406Y L3, and 66 nucleotide differences compared with the M982B L7 gene. These differences in the DNA sequence of the lst gene result in 16 and 17 amino acid differences in the protein gene product including the c-myc peptide tag (expected Mr, 44,000).

Expression of the Sialyltransferase Gene—We could easily detect enzyme activity in E. coli carrying pNST plasmids, and this expression of the lst gene depended on the vector derived promoter because there was no detectable enzyme activity when the gene's orientation was inverted. There was at least 30-fold more enzyme activity from the pNST-01 containing clones compared with the MC58 L3 and 406Y L3, respectively. The gene result in 16 and 17 amino acid differences in the protein when compared with the MC58 L3 and 406Y L3, respectively. However, the expression of the lst gene was not high enough to permit simple instrumental in successful screening of clones expressing the N
Sialyltransferases from Neisseria

Two substrate concentrations were used to assay enzyme activity from both sources. The relative specific activities were calculated from the percent conversion of the substrate to product using the CE assay. The assays were performed so that similar percent conversions are compared for both sources of enzyme.

TABLE II
Comparison of acceptor specificities of \(N.\) meningitidis \(\alpha\)-2,3-sialyltransferase

| Enzyme source | N-acetyllactosamine\(^a\) | Lactose | Galactose |
|---------------|-----------------|--------|--------|
|               | 0.2 mM  | 1.0 mM | 0.2 mM | 1.0 mM | 0.2 mM | 1.0 mM |
| MC58 L3       | 0.70\(^b\) | 1.45   | 0.11   | 0.45   | 0.006  | 0.033  |
| pNST-01       | 23.9    | 46.8   | 4.2    | 15.4   | 0.17   | 0.84   |

\(^a\) The acceptors used in this experiment were all FCHASE-aminophenyl-glycosides.
\(^b\) The activity values are in milliunits/mg of protein.

m. meningitidis \(\alpha\)-2,3-sialyltransferase. The assay uses a glycosyltransferase acceptor, which is easy to synthesize, and does not require specially constructed CE equipment as has been previously described for the ultrasensitive detection of glycosyltransferase reaction products (15). The acceptors used in this study were made from widely available glycosides, and fluorophores and the CE equipment used was commercially available. We were able to reliably detect attomole \((10^{-18} \text{ mol})\) quantities of reaction products, which was more than adequate for screening for \(\alpha\)-2,3-sialyltransferase expression.

The \(lst\) gene from MC58 L3 occurs between two genes unrelated to LOS synthesis, isocitrate dehydrogenase and cytochrome \(c\), and is not part of a LOS synthesis operon unlike other \(N.\) meningitidis LOS glycosyltransferases (8). This is similar to the situation with the \(E.\) coli and \(N.\) meningitidis \(\alpha\)-2,8-polysialyltransferase involved in capsule biosynthesis, although these genes are adjacent to the CMP-Neu5Ac synthetase (16). It is interesting to speculate that the \(lst\) gene is found on its own as the result of a transposition event, although we have no evidence for insertion elements or transposon-like sequences flanking the gene. Sequence analysis and database comparisons showed this gene to be distinct from both the mammalian \(\alpha\)-2,3-sialyltransferase family, the bacterial \(\alpha\)-2,8-sialyltransferase family, and the bacterial 3-deoxy-\(\alpha\)-D-manno-octulosonic acid transferases, which transfer a related sugar also from a CMP donor. The \(lst\) gene product was, however, shown to be similar to the \(lsg\)-ORF2 gene product from \(H.\) influenzae. Although \(lsg\)-ORF2 has been demonstrated to be involved in LOS biosynthesis, it may not encode an \(\alpha\)-2,3-sialyltransferase because it was reported to be involved in the expression of a Gal-GlcNAc LOS epitope (17). For the cloning of the \(N.\) gonorrhoeae gene, the F62 strain was used because it has been studied in relation to the role LOS sialylation plays in pathogenesis and because many LOS glycosyltransferases have been shown to be common to both species (8). An examination of the gene derived from \(N.\) gonorrhoeae F62 shows only a small number of differences, which is similar to other LOS biosynthesis gene comparisons from \(N.\) meningitidis and \(N.\) gonorrhoeae (8).

We have observed that the activity from \(N.\) meningitidis extracts is associated with the membrane fraction. The protein encoded by the \(lst\) gene appears to have an uncleavable signal peptide, and computer aided prediction programs suggest that the sialyltransferase is an integral inner membrane protein (14). However, the original papers describing the sialyltransferase activity from both \(N.\) meningitidis and \(N.\) gonorrhoeae suggest that the sialyltransferase would be an outer membrane protein on the basis that the enzyme activity is extracted from whole cells by Triton X-100 (5, 6). We have not yet fully purified the recombinant product, but the predicted size of the immunotagged protein is slightly larger than that we observed by SDS-PAGE. The difference between the observed and the expected \(M_\text{r}\) is less than 7% and is within the accuracy of SDS-PAGE. However, the possibility exists that the recombinant protein is truncated at the amino-terminal end, which would result in the loss of the predicted noncleavable leader sequence. Experiments are in progress to determine if the \(lst\) protein is expressed intact in \(E.\) coli.

That this gene functions in the sialylation of LOS is inferred from an examination of \(N.\) meningitidis M982B L7, which appears to be a natural sialyltransferase mutant. The sialyltransferase gene derived from this L7 strain contains a frameshift mutation at nucleotide 454 that renders it inactive in the recombinant plasmid carrying it, which agrees with our observation that sialyltransferase activity cannot be detected in M982B cells. This frameshift is a deletion of a T residue, which is different from the G-tract frameshift mutations observed in the phase variable \(lgt\) genes from \(N.\) meningitidis and \(N.\) gonorrhoeae (8). This strain produces the same lacto-N-neotetraose as the L3 strains do but does not sialylate its LOS.\(^3\) The acceptor specificity for the L3 enzyme with synthetic acceptors shows a strong preference for N-acetyllactosamine over lactose or galactose (Table II). Also the product of the reaction using enzyme from \(N.\) meningitidis and FCHASE-LacNAc acceptor was unequivocally determined by NMR to be FCHASE-\(\alpha\)-2,3-sialyl-N-acetyllactosamine.

The expression level of the recombinant gene is being optimized, but it should be pointed out that the level of enzyme activity we have produced is approximately 50 units/liter of culture, based on assays with the FCHASE-LacNAc acceptor. Our expression levels are as high as those reported for mammalian sialyltransferases being overexpressed in insect cell cultures (18). We anticipate that optimization of expression of the \(lst\) gene in \(E.\) coli will yield substantially more enzyme activity than is produced with the mammalian gene constructs. This will be an important improvement for large scale chemienzymatic synthesis of \(\alpha\)-2,3-sialylated oligosaccharides.

We have shown conclusively that we have cloned the \(\alpha\)-2,3-sialyltransferase gene from the important mucosal pathogens \(N.\) meningitidis and \(N.\) gonorrhoeae. The availability of this gene will enable defined mutants to be constructed in both species in order to determine the role that LOS sialylation plays in the pathogenesis of these organisms, and the regulation of this important virulence factor can now be studied. The availability of large amounts of an \(\alpha\)-2,3-sialyltransferase for enzymatic synthesis will help elucidate the role of this important modification of many oligosaccharide structures.

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