Sialylation of Lipooligosaccharide Cores Affects Immunogenicity and Serum Resistance of Campylobacter jejuni

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Three genes involved in biosynthesis of the lipooligosaccharide (LOS) core of Campylobacter jejuni MSC57360, the type strain of the HS:1 serotype, whose structure mimics GM2 ganglioside, have been cloned and characterized. Mutation of genes encoding proteins with homology to a sialyl transferase (cstII) and a putative N-acetylmannosamine synthetase (neuCl), part of the biosynthetic pathway of N-acetylneuraminic acid (NeuNac), have identical phenotypes. The LOS cores of these mutants display identical changes in electrophoretic mobility, loss of reactivity with cholera toxin (CT), and enhanced immunoreactivity with a hyperimmune polyclonal antiserum generated against whole cells of C. jejuni MSC57360. Loss of sialic acid in the core of the neuCl mutant was confirmed by fast atom bombardment mass spectrometry. Mutation of a gene encoding a putative β-1,4-N-acetylgalactosaminyltransferase (Cgt) resulted in LOS cores intermediate in electrophoretic mobility between that of wild type and the mutants lacking NeuNac. Loss of reactivity with CT, and a reduced immunoreactivity with hyperimmune antiserum. Chemical analyses confirmed the loss of N-acetylgalactosamine (GalNac) and the presence of NeuNac in the cgt mutant. These data suggest that the Cgt enzyme is capable of transferring GalNac to an acceptor with or without NeuNac and that the Cst enzyme is capable of transferring NeuNac to an acceptor with or without GalNac. A mutant with a nonsialylated LOS core is more sensitive to the bactericidal effects of human sera than the wild type or the mutant lacking GalNac.

Campylobacter jejuni and Campylobacter coli are among the most prevalent causes of bacterial diarrhea in the world (15, 31). These organisms are antigenically complex, as demonstrated by the fact that there are >70 serotypes based on heat-stable (HS) antigens (34) and >100 serotypes based on the heat-labile serotyping scheme (26). Campylobacters contain sialic acid moieties both in lipooligosaccharide (LOS) core structures (3–6, 29) and in posttranslational modifications on flagellin (9).

Structural analyses of a limited number of campylobacter strains has revealed LOS-like variability in the outer core (28, 29). Moreover, the outer cores of strains from multiple serogroups contain sialic acid moieties in structures which mimic human gangliosides. This molecular mimicry is thought to result in an autoimmune response responsible for the association of some campylobacter serotypes with Guillain-Barré syndrome (GBS) (1, 28, 29). However, the biological function of sialylated LOS to pathogenesis of gastroenteritis by C. jejuni has not been examined experimentally.

Campylobacter jejuni spp. are capable of endogenous biosynthesis of sialic acid (3–6, 9, 29). The genome of C. jejuni NCTC 11168 contains multiple genes which encode proteins with similarity to prokaryotic enzymes involved in biosynthesis of sialic acid, N-acetylneuraminic acid (NeuNac) (33). For example, NCTC 11168 has three copies of genes encoding sialic acid transferase activities and a putative N-1,3-acetylgalactosaminyltransferase (GalNac transferase) in an HS:19 isolate from a GBS patient (16). Herein, we describe a set of genes responsible for NeuNac biosynthesis in C. jejuni MSC57360, the type strain of the HS:1 serogroup, which has been shown to contain an LOS core which mimics GM2 ganglioside in structure (5), as seen in Fig. 1A. Moreover, we demonstrate that loss of NeuNac from the LOS core of MSC57360 affects the immunogenicity of the core and the serum sensitivity of the bacterium.

MATERIALS AND METHODS

Strains and growth conditions. C. jejuni MSC57360 (5) was a gift from John Penner, University of Toronto. C. jejuni strains were routinely grown on Mueller-Hinton (MH) agar, supplemented with kanamycin (50 μg/ml) or chloramphenicol (15 μg/ml) when appropriate at 37°C in a microaerobic environment. Escherichia coli XL-1 Blue was the host for λ ZAP Express (Stratagene, La Jolla, Calif.). The library was probed with a PCR product specific for cj1142 of NCTC 11168 (see below) which had been purified by agarose gel electrophoresis and labeled by random priming (Boehringer Mannheim, Indianapolis, Ind.) with [32P]dCTP (New England Nuclear, Boston, Mass.). Positive clones were plaque purified, rebighthed, and, once a pure population of positive phage was obtained, excised to the phagemid pBK-CMV, according to the instructions of the manufacturer.

The primers used to amplify the neuCl gene of MSC57360, designed based on the sequence of cj1142 (33), were NEU2-F, 5'-GGTGTATAGGTTGGAGCTTTAGCCTG-3', and NEU2-B, 5'-GTCAGTCTCACATCGTCTGTGAACC-3'. The PCR conditions used were 30 cycles of 94°C for 1 min, 49°C for 1 min, and 72°C for 1 min. The 630-bp product was sequenced using the same primers to confirm the identity of the product.

DNA sequence analysis. Plasmid DNAs were sequenced on both strands using terminator chemistries and Taq cycle sequencing kits from Perkin-Elmer Ap-
FIG. 1. (A) Structure of the LOS core of MSC57360 (5). Abbreviations: PE-A, O-phosphatic acid; KDO, 3-deoxy-D-manno-octulosonic acid; LDPhe, 1-glycerophospho-D-manno-heptose; Glc, glucose; Gal, galactose; GalNAc, N-acetylgalactosamine; NeuNAc, N-acetylneuraminic acid. (B) Sialic acid locus of MSC57360. The lengths of the following ORFs were as indicated: cst, 881 bp; neuB1, 1,113 bp; and neuC1, 1,113 bp. The position of insertion of a chloramphenicol resistance (Cmr) cassette is indicated by the arrows below the line. The insertion into neuC1 was constructed by insertion of the Cmr cassette into a unique NdeI site which is located 118 bp into the ORF. The insertions into cst and cst-neuC1 were constructed by in vitro transposition, and the position was determined by DNA sequence analysis, as described in Materials and Methods. The position of the insertion into cst was 12 bp into the ORF, and the insertion into the cst-neuC1 gene was located 396 bp into the ORF. In all three mutants the Cmr cassette was inserted in the same orientation as the gene is transcribed.
clones are summarized in Table 1. The first gene in the operon encodes a predicted protein of 35.1 kD with 100% identity to cj1140 from NCTC 11168, which was annotated by Parkhill et al. (33) as an unknown. However, the gene product also shows 53% identity and 69% similarity to a predicted protein encoded by the cstII gene of C. jejuni OH4384, which was shown by Gilbert et al. to be a bifunctional sialyl transferase, capable of adding NeuNAc by an α-2,3 linkage to D-galactose and by an α-2,8 linkage to NeuNAc (16). The MSC57360 protein also shows 32% identity and 33% similarity to the corresponding enzyme from OH4384 (16).

The second ORF, which overlaps cst by 16 bp, encodes a predicted protein of 38.4 kDa that differs from cj1141 of NCTC 11168 by only 2 amino acids. This protein was designated neuB1 by Parkhill et al. (33) based on its homology to the NeuB (43), from SiaC (11, 41). The corresponding enzyme from OH4384 (16) shows 76% identity and 86% similarity to the corresponding protein in C. jejuni OH4384 (16).

ORF3 starts at the same base pair that ORF2 stops at and encodes a predicted protein of 42.7 kDa which shows 100% identity to cj1142 or NeuC1, designated by Parkhill et al. (33) as a putative N-acetylgalactosamine(GlcNAc)-6-phosphate 2-epimerase–GlcNAc-6-phosphatase based on the high level of homology to the corresponding enzyme, SiaA (11, 35, 41), in N. meningitidis (43% identity and 63% similarity). This enzyme is involved in biosynthesis of ManNAc, the precursor of NeuNAc (35). The MSC57360 and NCTC 11168 proteins show 65% identity and 76% similarity to the corresponding protein in OH4384 (16).

The start of ORF4 overlaps with the stop codon of ORF3 and encodes a predicted protein of 62.5 kDa. This protein shows 97% identity with cj1143 from NCTC 11168 (33) over the full length (536 amino acids). Protein cj1143 was annotated as a CMP-NeuNAc synthetase by Linton et al. (25). However, the homology of cj1143 and ORF4 of MSC57360 to known CMP-NeuNAc synthetases is limited to the carboxy-terminal 218 amino acids. This region also shows 67% identity and 80% similarity to a putative CMP-NeuNAc synthetase described in OH4384 (16) and 38% identity and 57% similarity to a known CMP-NeuNAc synthetase from Hemophilus ducreyi (42). The N-terminal 280 amino acids of ORF4 shows 44 to 45% identity and 54 to 55% similarity to two β-1,4-N-acetylgalactosaminyl-transferase (Cgt) enzymes (GalNAc transferases) from OH4384 and another C. jejuni HS19 isolate (16). Thus, this ORF in both MSC57360 and NCTC 11168 appears to represent a fusion of the cgt and neuA1 genes.

**Insertional mutagenesis of MSC57360 LOS genes.** A Cm<sup>R</sup> cassette (50) was inserted as a Smal-ended fragment into a unique Ndel site within neuC1 which had been blunted by treatment with Klenow enzyme. This plasmid, called pMSC203::Cm was used to transform MSC57360. Subsequent mutations into cst and cgt-neuA were generated in E. coli DH5α by in vitro transposition of a Cm<sup>R</sup> cassette (50) as described in Materials and Methods. The position and orientation of the transposon insertions into individual plasmids was determined by DNA sequence analysis, and selected insertions were transformed into MSC57360. All C. jejuni transformants were characterized by PCR to confirm that the insert had integrated via double crossover (data not shown).

Proteinase K-treated whole cells from MSC57360 and the mutants were electrophoresed on Tricine gels and silver stained to visualize LOS cores. The results, shown in Fig. 2A, indicate that the mobility of the cores of cst (lane 2) and neuC1 (lane 3) mutants were identical to one another but were reduced in apparent M<sub>r</sub> compared to the wild type (lane 1). The cgt-neuA mutant (lane 4) displayed an intermediate mobility.

![FIG. 2. Comparison of LOS of MSC57360 and mutants. Proteinase K-digested whole-cell preparations were electrophoresed on 16% Tricine gels and silver stained (A), reacted with CT (final concentration, 1 μg/ml) (B), or immunodetected with polyclonal rabbit antiserum against whole cells of MSC57360 (final dilution, 1:500) (C). Lane 1, MSC57360; lane 2, MSC57360 cst; lane 3, MSC57360 neuC1; lane 4, MSC57360 cgt. The apparent M<sub>r</sub> of the LOS core of wild-type MSC57360 on Tricine gels is approximately 9.2 kDa.](image-url)
between that of the wild type and the cst and neuC1 mutants. Figure 2B shows the reaction of the LOS cores with CT; all three mutants have lost reactivity with CT. Similar loss of reactivity with CT was observed with purified LOS (data not shown). Figure 2C shows an immunoblot of the whole-cell digests which have been immunodetected with a polyclonal rabbit antiserum generated against whole cells of MSC57360. The results indicate that the cst (lane 2) and neuC1 (lane 3) mutants showed enhanced immunoreactivity compared to wild-type MSC57360 (lane 1). The LOS core of the cst-neuA mutant, however, was not detected at the antibody dilution used (lane 4).

To confirm that the insertion into neuC1 was not exerting a polar effect on cst-neuA, a Km' shuttle plasmid (pMSC1420) containing the cst, neuB1, and neuC1 genes was transferred into the neuC1 mutant. As seen in Fig. 3, the mobility of the core was restored to that of the wild type, CT binding was restored, and the immunoreactivity with the anti-MSC57360 antibody was reduced (lanes 3).

**Chemical characterization of the LOS core of the neuC1 and cst-neuA mutants of MSC57360.** Upon methanolysis followed by peracetylation of LOS of wild-type MSC57360 and cst-neuA, the peracetylated methyl ketoside methyl ester derivative of NeuNAc was detected by GLC and combined GLC-MS. The NeuNAc derivative from the LOS was identical in all parameters in GLC-MS to authentic NeuNAc which under-went the same derivatization procedure. Unlike these LOSs, NeuNAc was not detected in the neuC1 mutant LOS when a colorimetric assay was used or when more-sensitive detection by GLC-MS was utilized. Furthermore, to confirm the loss of NeuNAc from the LOS of this strain, core oligosaccharides were liberated from LOSs of wild-type MSC5730 and the neuC1 mutant, methylated, and subsequently analyzed in FAB-MS. As shown in Fig. 4, the permethylated core oligosaccharides of wild-type MSC5730 possessed a pseudomolecular ion, m/z = 2596 [M + H]+, and the mass spectrum included daughter ions indicative of sialylation, particularly m/z = 376. In contrast, the mass spectrum of the neuC1 mutant lacked the latter ion, and the pseudomolecular ion, m/z = 2234 [M + H]+, was sufficiently less because of the absence of NeuNAc. The results, therefore, support the loss of NeuNAc from the neuC1 mutant.

**Effect of sia mutations of MSC57360 flagellin.** Flagellins of *Campylobacter* spp. have been shown to contain sialic acid, which affects the glycoform pattern in IEF gels (9, 18). Flagellins were purified from MSC57360 wild type and the cst, neuC1, and cst-neuA mutants, and the IEF patterns were examined. Figure 5 shows that there was no difference in the IEF pattern of flagellin from wild-type MSC57360 (lane 3) and the neuC1 mutant (lane 4). Similarly, there was no difference in the absence of an N-acetylhexosamine (HexNAc) residue. Consistent with this, the mass spectrum lacked the daughter ion, m/z = 260 but contained daughter ions indicative of sialylation, including m/z = 376. Moreover, NeuNAc was detected by GLC-MS analysis of cst-neuA mutant LOS after methanalysis and peracetylation as described above. Thus, the core oligosaccharide of cst-neuA mutant LOS lacks terminal NeuNAc but is sialylated.

**FIG. 3.** Complementation of MSC57360 neuC1 in trans. Plasmid pMSC1420 was conjugally mobilized from *E. coli* DH5α (RK212.2) into MSC57360 neuC1. Proteinase K-digested whole-cell preparations were electrophoresed on 16% Tricine gels and silver stained (A), reacted with CT (final concentration, 1 μg/ml) (B) or immunodetected with polyclonal rabbit antiserum against whole cells of MSC57360 (final dilution, 1:300) (C). Lane 1, MSC57360; lane 2, MSC57360 neuC1; lane 3, MSC57360 neuC1 (pMSC1420).

**FIG. 4.** Analysis of positive-ion FAB-MS for permethylated core oligosaccharides from LOS of *C. jejuni* MSC57360 and mutants. Numbers refer to m/z values for ions. Abbreviations: HexNAc, N-acetylhexosamine; Hex, hexose; Hep, heptose; Kdo, 3-deoxy-D-manno-2-octulosonic acid. The pseudomolecular ion and daughter ions observed for the oligosaccharide of MSC57360 are indicated by a single asterisk, whereas those of mutants in neuC1 and cst-neuA are indicated by two and three asterisks, respectively. The ion for NeuNAc, indicated by a single pound sign, was absent from the neuC1 mutant, and that for HexNAc indicated by two pound signs was absent in the cst-neuA mutant.

**FIG. 5.** Comparison of IEF patterns of flagellins of VC167 T2 and MSC57360. Flagellins were electrophoresed on IEF gels of pH 4 to 6 and stained with Coomassie blue. Lane 1, VC167 T2; lane 2, VC167 T2 pimB1; lane 3, MSC57360; lane 4, MSC57360 neuC1.
the IEF pattern of flagellins isolated from either the cst or cgt-neuA mutants (data not shown), indicating that these MSC57360 genes are not involved in biosynthesis of the post-translational modifications of flagellin. Flagellin from C. coli VC167 and a pmB mutant encoding a CMP-NeuNAc synthetase (18) are shown for comparison. Interestingly, the wild-type flagellins from VC167 and MSC57360 display markedly distinct IEF patterns, suggesting differences in the posttranslational modifications of these proteins.

Loss of sialic acid in LOS results in increased serum sensitivity. Figure 6 compares the sensitivities of wild-type MSC57360 and the neuC1 and cgt-neuA mutants to normal human sera. Bacteria were incubated with normal human sera and the same serum which had been heated to inactivate complement. Bacterial counts were determined at 0, 30, and 60 min of incubation. (Results are given as means ± standard errors.) After 30 min of incubation the cgt-neuA mutant showed serum sensitivity (70% ± 10.0% survival) comparable to that of the wild type (60% ± 3.0% survival; P = 0.07), but the survival of the neuC1 mutant (27% ± 8.0%) was significantly reduced compared to the wild type (P = 0.0001). After 60 min of incubation, survival of the wild type and cgt-neuA was reduced to 37% ± 12.0% and 44% ± 30.0%, respectively. Survival of the neuC1 mutant was 9% ± 6.0% (P = 0.01). Heat inactivation of the serum pools resulted in loss of all bactericidal activity (data not shown).

**DISCUSSION**

Sialic acid is an important surface component of a number of bacterial pathogens. The similarity of the polysialic acid capsules of E. coli K1 and meningococci with the embryonic form of the neural cell adhesion molecule is thought to be responsible for the poor immunogenicity of these neuropathogens (13). Moreover, sialylated capsules and LOS are known to render bacteria resistant to complement killing (14, 36, 39, 44–47) and can affect bacterial interactions with neutrophils (40, 47) and epithelial cells (44, 45). Although considerable attention has focused on the relationship of the sialylated LOS cores of C. jejuni and the development of GBS (1, 28), the function of sialylation in the pathogenesis of diarrheal diseases has not been considered. In an effort to begin to elucidate this role, we have generated mutations affecting the core of the type strain of the HS:1 serogroup, which has a defined LOS core structure with GM2 ganglioside mimicry.

The genetic locus of MSC57360 described here is involved in biosynthesis of LOS cores, as are the corresponding genetic loci described for HS:19 and HS:2 strains (16, 25). Mutation of the neuC1 and cst genes resulted in identical phenotypes of LOS cores, each with the same change in electrophoretic mobility, loss of reactivity with CT, and enhanced immunoreactivity with a polyclonal antibody against whole cells of the strain. Chemical analyses of the core of the neuC1 mutant confirmed the loss of NeuNAc. The loss of sialic acid in the core of the cst mutant suggests that, unlike the situation described in the GBS isolate OH4384, MSC57360 does not contain a second copy of a sialyl transferase with α-2,3-sialyltransferase activity. Moreover, BLASTP analysis suggests that NCTC 11168 also contains a single sialyl transferase with homo-logy to those described in OH4384 (16).

Both C. jejuni NCTC 11168 and MSC57360 have a gene which appears to be a fusion of genes encoding Cgt and a CMP-NeuNAc synthetase. Although this ORF was annotated by Parkhill et al. as a CMP-NeuNAc synthetase (33), the protein appears to function in MSC57360 as a GalNAc transferase. The core mobility displayed by a mutant in this gene was intermediate between that of the wild type and the cst and neuC1 mutants, suggesting that the cst mutant core was still sialylated, and FAB-MS analyses confirmed the loss of GalNAc and the presence of sialic acid. This is in contrast to the data of Gilbert et al. (16) who reported that the galactosyltransferase activity of Cgt from OH4384 was specific for a sialylated acceptor. In MSC57360 it appears that the Cgt enzyme can transfer GalNAc to a nonsialylated acceptor, and, conversely, Cst can transfer NeuNAc to a core lacking GalNAc. If sialic acid were added to a precursor structure (Fig. 7A), there would exist an intermediate structure which is identical to the core of the type strain of HS:2 (6) (Fig. 7B). This structure, which is also the predicted core of the cst mutant, would be expected to be poorly immunogenic. If the GalNAc were added to the core first, there would be no ganglioside mimicry in the intermediate (Fig. 7C), and it would be expected to be immunogenic, similar to the core of the cst mutant. The presence of antibodies in polyclonal antisera generated against whole cells of MSC57360 suggests that such immunogenic intermediate structures are present in low amounts in the population of LOS cores.

Interestingly, mutation of ORF4, which is a fusion of cgt (16) and neuA, results in the loss of GalNAc but not NeuNAc from the LOS core. This suggests that the fusion protein has either lost CMP-NeuNAc synthetase activity or that there are additional copies of genes encoding enzymes with the same function. Indeed, NCTC 11168, in addition to containing cgt-neuA (neuA1), contains two other copies of neuA alleles, cj1311 (neuA2) and cj1331 (neuA3 or pmB). The neuA3 or pmB allele has been shown to be involved in posttranslational modification of flagellin of C. coli VC167 (17) (Fig. 5), but the role of this gene in LOS biosynthesis in VC167, whose core is uncharacterized, remains open. Clearly, the role of the multiple neuA alleles in Campylobacter spp. requires additional study.

The presence of NeuNAc in the LOS core of MSC57360 results in decreased immunogenicity of the core and increased resistance to serum killing by complement. In Neisseria the presence of sialic acid on LOS also results in serum resistance but reduces the ability of the bacteria to be internalized into some eukaryotic cells (44, 45). There is a tremendous range in the ability of different strains of C. jejuni to be internalized into intestinal epithelial cells (22–45, 32) as well as differences in the behavior of different strains in various animal models of virulence (7; D. Burr and P. Guerry, unpublished data). There are no reports of which we are aware on the virulence of...
MSC57360 in animal models, but the strain invades INT407 cells at levels below those of E. coli K-12 (data not shown). However, having established the function of these genes in a strain of known LOS core structure, we are now examining the effect of LOS sialylation on pathogenesis of virulent strains of C. jejuni.

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FIG. 7. Schematic of alternate pathways in the synthesis of the core of MSC57360 (D). Shown are different intermediate structures which could be generated during biosynthesis of the core of MSC57360 depending upon the order in which the sialyl transferase, Cst, and the GalNAc transferase, Cgt, react with the precursor structure (A). Structure B is the same as the core structure of the type strain of HS:2 (B). Structure C corresponds to the predicted core of the cst mutant.


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