Commonality and Biosynthesis of the O-Methyl Phosphoramidate Capsule Modification in Campylobacter jejuni*§

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In this study we investigated the commonality and biosynthesis of the O-methyl phosphoramidate (MeOPN) group found on the capsular polysaccharide (CPS) of Campylobacter jejuni. High resolution magic angle spinning NMR spectroscopy was used as a rapid, high throughput means to examine multiple isolates, analyze the cecal contents of colonized chickens, and screen a library of CPS mutants for the presence of MeOPN. Sixty eight percent of C. jejuni strains were found to express the MeOPN with a high prevalence among isolates from enteritis, Guillain Barré, and Miller-Fisher syndrome patients. In contrast, MeOPN was not observed for any of the Campylobacter coli strains examined. The MeOPN was detected on C. jejuni retrieved from cecal contents of colonized chickens demonstrating that the modification is expressed by bacteria inhabiting the avian gastrointestinal tract. In C. jejuni 11168H, the cj1415-cj1418 cluster was shown to be involved in the biosynthesis of MeOPN. Genetic complementation studies and NMR/mass spectrometric analyses of CPS from this strain also revealed that cj1421 and cj1422 encode MeOPN transferases. Cj1421 adds the MeOPN to C-3 of the β-D-GalNAc residue, whereas Cj1422 transfers the MeOPN to C-4 of D-glycero-D-gulo-heptopyranose. CPS produced by the 11168H strain was found to be extensively modified with variable MeOPN, methyl, ethanolamine, and N-glycolyl groups. These findings establish the importance of the MeOPN as a diagnostic marker and therapeutic target for C. jejuni and set the groundwork for future studies aimed at the detailed elucidation of the MeOPN biosynthetic pathway.

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–3 and Figs. 1 and 2.

The abbreviations used are: CPS, capsular polysaccharide; MeOPN, O-methyl phosphoromimidate CH₂OP(O)(NH₂)(OR); CE-ESI/MS, capillary electrophoresis electrospray ionization mass spectrometry; HMQC, heteronuclear multiple-quantum coherence; HMPTOXY, heteronuclear multiple-quantum coherence total correlation spectroscopy; HR-MAS NMR, high resolution magic angle spinning nuclear magnetic resonance spectroscopy; HSQC, heteronuclear single-quantum coherence; NOESY, nuclear Overhauser effect spectroscopy; Kan, kanamycin.

Campylobacter jejuni is the leading cause of bacterial foodborne gastroenteritis, a causative agent of child morbidity in underdeveloped countries and an antecedent to the Miller-Fisher and Guillain-Barré neuropathies (1–5). Furthermore, C. jejuni now surpasses Salmonella, Shigella, and Escherichia in some regions as the primary cause of bacterial gastrointestinal disease (6–8). Because the number of reported C. jejuni infections is increasing worldwide, there is growing interest to identify virulence mechanisms associated with this mucosal pathogen as a critical step toward the development of control strategies.

The capsular polysaccharides (CPS)5 produced by C. jejuni are known to be important virulence factors that are involved in colonization and invasion (9, 10). CPS expression was shown to be necessary for diarrheal disease in ferrets, mediating mouse and chicken colonization, increasing resistance to human serum, as well as increasing adherence and invasion of human epithelial cells (9). The CPSs produced by C. jejuni are the major antigenic component of Penner’s serotyping system (10). There are now more than 60 serostrains described for this bacterium. Although not every strain has been examined, it is thought that each one produces a CPS having a different structure (11, 12). Furthermore, there can be extensive phase-variable structural modifications such as the incorporation of methyl, ethanolamine, and aminoglycerol groups on CPS sugars (13–15). It is thought that these extensive modifications may allow the bacterium to evade host defenses (14, 15).

The most unusual CPS modification is the O-methyl phosphoromimidate CH₂OP(O)(NH₂)(OR) (MeOPN) group, which is a labile phosphorylated structure. Nitrogen-phosphorus bonds are rare in nature, and we reported the first example of such a structure produced by a bacterium in the CPS of C. jejuni NCTC11168 (HS:2) (13) (Fig. 1). Since this initial study, the G1 (HS:1), NCTC12517 (HS:19) and 81-176 (HS:23/26) strains of C. jejuni have been shown to produce MeOPN (14–16) and a...
related structure has been identified on the lipooligosaccharide of *Xanthomonas campestris*, a Gram-negative plant pathogen (17). Preliminary structural analyses of CPS from different *C. jejuni* serotypes suggested the presence of the MeOPN modification on a diverse range of CPS sugars (18). This observation prompted us to isolate and elucidate the CPS structure from the G1 and NCTC12517 strains. The G1 CPS was subsequently shown to have a [-4)-α-D-Galp-(1-2)-(R)-Gro-(1-P)]n repeating unit with two labile β-D-fructofuranose branches at C-2 and C-3 of Gal. Each fructofuranose was further substituted at C-3 with MeOPN groups (14). Similarly, the NCTC12517 CPS was shown to have a [-4)-β-D-GlcA6NGro-(1–3)-β-D-GlcNAc-(1-P)]n repeating unit with a labile α-L-sorbofuranose branch at C-2 of GlcA and an MeOPN at C-4 of GlcNAc (15). For both strains, the keto sugars and MeOPN groups were found to be nonstoichiometric and were hypothesized to contribute to the overall structural heterogeneity of the CPS. Furthermore, the MeOPN was found to be variably methylated in the NCTC12517 strain thereby adding additional variability to an already structurally heterogeneous CPS. Most recently, Kanipes and co-workers (16) demonstrated that the 81-176 strain also has a MeOPN CPS modification and provided evidence to show that it most likely is found at C-2 of the galactose residue.

In a recent study, the CPS biosynthetic regions for selected strains of *C. jejuni* were sequenced, including 176.83 (HS: 41), 81-176, ATCC43456 (HS:36), CCUG10954 (HS:23), NCTC12517, and G1 (18). Comparison of the CPS sequences for the NCTC12517, 176.83, and G1 strains to the genome-sequenced NCTC11168 strain provided evidence for multiple mechanisms of CPS variation, including exchange of capsular genes by horizontal transfer, gene duplication, deletion, fusion, and contingency gene variation. Interestingly, the study uncovered the presence of a highly conserved gene cluster (cj1415–cj1420) within those strains that produce the MeOPN CPS modification (NCTC11168, NCTC12517, G1, 81-176). Because these genes have no apparent role in sugar biosynthesis and because their presence coincides with the presence of MeOPN on CPS sugars, it was hypothesized that they might be involved in MeOPN biosynthesis. Furthermore, in our recent study we demonstrated that 1H and one-dimensional 1H-31P HSQC spectra were acquired using the standard Varian HSQC pulse sequence with one-dimensional spectra representing the first increment of the standard HSQC experiment. All 1H NMR spectra were referenced to an internal 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt standard (δ1H 0.00 ppm).

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains and Growth Conditions—* *C. jejuni* NCTC11168 (HS:2) and its motile variant, 11168H (HS:2) were routinely grown on Mueller Hinton (MH) agar at 37 °C under microaerobic conditions (85% N2, 10% CO2, 5% O2). The NCTC11168 strain was originally isolated from a case of human enteritis (20) and later sequenced by Parkhill et al. (21). To select for kanamycin (Kan)-resistant mutants, Kan was added to the medium at a final concentration of 30 μg/ml. For large scale extraction of CPS, 6 liters of bacteria were grown in brain heart infusion broth (Difco) under microaerobic conditions at 37 °C for 24 h with agitation at 100 rpm. Bacterial cells were then harvested by centrifugation (9,000 × g for 20 min) and placed in 70% ethanol. Cells were removed from the ethanol solution by centrifugation (9000 × g for 20 min), and the bacterial pellet was refrigerated until extraction.

**High Resolution Magic Angle Spinning (HR-MAS) NMR Spectroscopy**—Bacterial cells were prepared and analyzed by HR-MAS NMR spectroscopy as described previously (13, 19). HR-MAS NMR experiments were performed using a Varian Inova 500-MHz (1H) spectrometer (Varian, Palo Alto, CA) equipped with a Varian 4-mm indirect detection gradient nano-NMR probe with a broadband decoupling coil. Samples were spun at 3 kHz, and spectra were recorded at ambient temperature (23 °C). HR-MAS NMR experiments were generally performed with suppression of the HOD signal using presaturation as described previously (13). 1H NMR spectra of bacterial cells were acquired using the Carr-Purcell-Meiboom-Gill pulse sequence (90-(τ–180–τ)2 acquisition) to remove broad signals originating from lipids and solid-like materials (22) and were typically obtained using 256 transients (11 min). The total duration of the Carr-Purcell-Meiboom-Gill pulse (n × 2τ) was 10 ms with τ set to 1/2MAS spin rate. One-dimensional 1H-31P HSQC spectra were acquired using the standard Varian HSQC pulse sequence with one-dimensional spectra representing the first increment of the standard HSQC experiment. All 1H NMR spectra were referenced to an internal 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt standard (δ1H 0.00 ppm).

*Bacterial Colonization of Specific Pathogen-free Leghorn Chicks*—The inoculum for each chick colonization experiment was prepared by harvesting *C. jejuni* 11168H cells, grown for 18 h into a 0.1 M (pH 7.4) phosphate-buffered saline solution (supplemented with 0.14 M NaCl and 0.002 M KCl). One-day-old specific pathogen-free chicks were orally gavaged with 300 μl of inoculum containing ~3 × 1010 bacterial cells. Because chicks typically do not consume feed during the first 48 h after hatching (23), cecal contents were analyzed 48 h post oral gavage to minimize the amount of particulate matter within the cecal contents that could potentially interfere with HR-MAS NMR. Furthermore, only adherent bacteria should be present at 48 h post oral gavage because the rate of passage through the gastrointestinal tract of leghorns is ~4 h (24). All chicks were euthanized by cervical dislocation according to the approved guidelines of the Canadian Council for Animal Care. Cecal contents were then serially plated onto Karmali agar (Oxoid, Ontario, Canada) and examined for the presence of *C. jejuni* and for MeOPN using HR-MAS NMR spectroscopy. In total,
the cecal contents from 39 chicks were examined (13 chicks from three independent experiments).

Construction of C. jejuni Site-specific Mutations—Mutants were constructed via insertion of a kan' cassette into unique sites present within appropriate pUC18-based recombinant plasmids from a random genomic library of C. jejuni (strain NCTC11168) that was constructed during the course of the genome sequencing project (21) (supplemental Table 1). The kan' cassette was inserted in a nonpolar orientation, and the derivatives were used for transformation of the C. jejuni 11168H strain via electroporation. Mutants were verified by PCR using gene-specific and kan'-specific primers. The cj1421/cj1422 double mutant was created through spontaneous insertion of the kan' cassette into both genes during construction of the cj1422 mutant because of the presence of identical regions for these genes. Integration of the kan' cassette within both genes was detected by PCR analysis using cj1421 and cj1422-specific primers and was later confirmed by sequencing.

Complementation Studies of the cj1421/cj1422 Double Mutant—cj1421 and cj1422 were PCR-amplified using chromosomal DNA obtained from the 11168H strain and high fidelity Taq polymerase (Accuprime Taq HiFi, 95 °C 15 s, 30 cycles of 95 °C for 15 s, 55 °C for 15 s, and 68 °C for 2 min) (Invitrogen) using the primers listed in supplemental Table 2. PCR fragments containing complete gene copies were then used for complementation studies using the pRED1 integrational expression vector according to Karlyshev and co-workers (25). To produce the delivery plasmids pRR1421 and pRR1422, a 0.7-kb gfp-SwaI/XbaI fragment of pRED1 was replaced with a 1.9-kb PCR product that was digested with PmeI/XbaI. As a precaution, pRR1421 and pRR1422 were sequenced because cj1421 and cj1422 contain homopolymeric G tracts that are prone to length variation (18). Following electroporation of the delivery constructs into the appropriate mutants, complemented strains were then used for complementation studies using the pRED1 integrational expression vector according to Karlyshev and co-workers (25).

![Diagram](image_url)
selected on blood agar plates and supplemented with Kan (50 µg/µl) and chloramphenicol (15 µg/µl). Integration of the cmr cj1421 and cmr cj1422 gene fusions into the rRNA gene cluster on the 11168H chromosome was confirmed by PCR as described previously (25).

Isolation and Purification of CPS—For large scale extraction of CPS, 6 liters of the C. jejuni 11168H wild type and the cj1421 mutant were grown in brain heart infusion broth under microaerobic conditions at 37 °C for 24 h with agitation at 100 rpm. Capsular polysaccharide was then isolated and purified using a gentle enzymatic method according to McNally et al. (14).

High Resolution NMR Spectroscopy of Purified CPS—For NMR spectroscopy of CPS isolated from the C. jejuni 11168H wild type and the cj1421 mutant, –3 mg of pure CPS was suspended in 200 µl of 99% buffered D₂O (50 mM NH₄HCO₃, pH 8.0) and placed in a 3-mm NMR tube. NMR experiments were performed using a Varian Inova 500 MHz (1H) spectrometer equipped with a Varian Z-gradient 3-mm triple resonance (1H, 13C, 31P) probe, or a Varian 600 MHz (1H) spectrometer equipped with a Varian 5-mm, Z-gradient triple resonance cryogenically cooled probe (cold probe). One-dimensional 31P spectra were acquired using a Varian Mercury 200-MHz (1H) spectrometer and a Nalorac 5-mm four nuclei probe. NMR experiments were typically performed at 25 °C with suppression of the HOD resonance at 4.78 ppm. Standard homo- and heteronuclear correlated two-dimensional pulse sequences from Varian were used for general assignments. Selective one-dimensional total correlation spectroscopy and NOESY experiments with a Z-filter were used for complete residue assignment as well as for measuring J_H,P coupling constants and nuclear Overhauser enhancements (26, 27). Proton and carbon resonances were referenced to an internal acetone standard (∆H 2.225 ppm, ∆C 31.07 ppm), whereas phosphorus signals were referenced to an external 85% phosphoric acid standard (∆P 0.00 ppm).

Mass Spectrometric Analyses of Purified CPS—CPS isolated from the C. jejuni 11168H wild type and the cj1421 mutant were mass-analyzed using in-source collision-induced dissociation CE-ESI/MS according to Li et al. (28) with a Prince system capillary electrophoresis instrument (Prince Technologies, Emmen, The Netherlands) coupled to a 4000 Qtrap spectrometer (Applied Biosystems/Sciex, Foster City, CA) via a microion spray interface. A sheath solution (isopropyl alcohol/methanol, 2:1) was delivered at a flow rate of 1 µl/min. Separations were
achieved on ~90 cm of bare-fused silica capillary (360 μm outside diameter × 50 μm inner diameter, Polymicro Technologies, Phoenix, AZ) using 15 mM ammonium acetate/ammonium hydroxide in deionized water, pH 9.0, containing 5% MeOH as the separation buffer. A voltage of 20 kV was typically applied during CE separation, and +5 kV was used as electrospray voltage. Mass spectra were acquired with dwell times of 3.0 ms per step of 0.1 m/z unit in full mass scan mode using an orifice voltage of +400 V. Fragment ions formed by collision activation of selected precursor ions with nitrogen in the RF-only quadrupole collision cell were then mass-analyzed by scanning the third quadrupole.

RESULTS

Commonality and Specificity of the MeOPN CPS Modification in Campylobacter—Assessing the commonality and specificity of MeOPN expression among Campylobacter isolates is important to determine the abundance of this CPS modification and its potential as a diagnostic marker and therapeutic target. Previous studies have shown that the MeOPN is readily detected on the surface of intact C. jejuni cells using HR-MAS 1H NMR spectroscopy (13–15, 19) making this technique amenable to the rapid, high throughput screening of multiple strains. The MeOPN can be considered as a unique NMR tag because signals arising from its –OCH3 protons are intense compared with other CPS signals and have a unique chemical shift and phosphorus scalar coupling. To assess MeOPN prevalence, intact cells from 63 C. jejuni strains and 18 Campylobacter coli strains were screened using HR-MAS 1H NMR, as well as 31P decoupled, 1H-31P HSQC HR-MAS NMR experiments. The C. jejuni strains were comprised of human and animal isolates; originated from a range of geographical locations that included Canada, the United States, Japan, the Netherlands, Brazil, and the United Kingdom; and are responsible for a variety of clinical presentations, including asymptomatic, enteritis, Guillain-Barré syndrome, Miller-Fisher syndrome, and septicemia (Table 1). Overall, 43 of 63 or 68% of the C. jejuni strains were found to express the MeOPN CPS modification. Interestingly, the MeOPN was observed for 82% of enteritis strains, 80% of Guillain Barré syndrome strains, and 100% of Miller-Fisher syndrome strains. In contrast, MeOPN was not detected for any of the 18 C. coli strains examined.

Detection of MeOPN from the Cecal Contents of Colonized Chickens—To assess the expression of the MeOPN within the natural avian host, a chicken model was developed using 1-day-old specific pathogen-free chicks that were inoculated with a high dose of C. jejuni 11168H cells. Fig. 2 is representative of the 13 cecal contents that were examined using HR-MAS NMR spectroscopy. The HR-MAS 1H NMR spectrum of in vitro plate-grown C. jejuni 11168H cells exhibited clear signals originating from two partially overlapped MeOPNs at δH 3.77 ppm and δH 3.79 ppm with phosphorus scalar couplings of approximately JHP 12.0 Hz (Fig. 2a, note that the partial overlap of the doublet signal from each MeOPN results in the appearance of three peaks). Signals originating from the anomeric protons of the CPS sugars β-d-Ribp (A1), α-d-GlcpA (B1), β-d-GlcpNac (C1), and d-glycero-α-l-gluco-heptopyranos (D1) were observed in agreement with the CPS structure reported for the NCTC11168 strain (Fig. 1 and Fig. 2a) (8, 13). Two anomeric signals were observed for residue C as a result of structural heterogeneity generated by the phase variable MeOPN group at C-3 of this sugar (13). Furthermore, a clear singlet was observed for the –OCH3 group (OMe) located at C-6 of residue D. In contrast, the HR-MAS 1H NMR spectrum of the cecal contents harvested from one of the C. jejuni colonized chicks was complicated by signals originating from cecal matter that
MeOPN CPS Modification in C. jejuni

overlapped with CPS signals (Fig. 2b). To specifically probe for the MeOPN, a one-dimensional, $^{31}$P decoupled, $^1$H-$^{31}$P HSQC HR-MAS NMR experiment was used that attenuates signals from cecal matter (15, 19). Using this experiment to examine plate-grown intact cells, two clear signals could be observed originating from both MeOPN groups at $\delta_{H}$ 3.77 and 3.79 ppm (Fig. 2a, inset). In a similar fashion, analysis of the cecal contents of C. jejuni colonized chicks revealed that both MeOPN signals could be readily detected in all samples examined (see Fig. 2b, inset, for a representative spectrum).

Identification of Genes Involved in MeOPN Biosynthesis and Transfer—To identify MeOPN biosynthesis genes, HR-MAS $^1$H NMR experiments were used to rapidly examine a library of CPS mutants for the presence of MeOPN (Fig. 3, shaded region). The HR-MAS $^1$H NMR spectrum of 11168H wild type cells showed the expected anomeric signals for CPS sugars, the $-$OCH$_3$ group located on residue D, as well as two partially overlapping doublet signals at $\delta_{H}$ 3.77 and 3.79 ppm originating from both MeOPN groups with $^{31}$P scalar couplings of approximately $J_{H,P}$ 12.0 Hz each (Fig. 3a). In contrast, MeOPN signals were not detected for the cj1415, cj1416, cj1417, and cj1418 mutants that clearly implicated these genes in the synthesis or transfer of MeOPN (Fig. 3, b–e). Of importance, all of the mutants examined produced CPS as indicated by the anomeric resonances that are observable within the HR-MAS $^1$H NMR spectra (Fig. 3, labels A1–D1). HR-MAS $^1$H NMR and one-dimensional $^1$H-$^{31}$P HSQC analyses of the cj1419 and cj1420 mutants revealed signals from both MeOPN groups thereby negating a role for these genes in MeOPN biosynthesis (Fig. 3f). In contrast, spectra for the cj1421 mutant showed the loss of the MeOPN signal at $\delta_{H}$ 3.77 ppm, whereas that for cj1422 revealed the loss of the MeOPN signal at $\delta_{H}$ 3.79 ppm thereby implicating the products of these genes in MeOPN biosynthesis or transfer (Fig. 3f).

Structure Elucidation of CPS Purified from C. jejuni 11168H—Based on these findings, it was concluded that cj1415, cj1416, cj1417, cj1418, cj1419, cj1420, cj1421, and cj1422 are involved in MeOPN biosynthesis or transfer.

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**FIGURE 5.** Mass spectrometric analysis of CPS purified from C. jejuni 11168H. a, CE-ESI-MS total ion chromatogram (positive ion mode, orifice voltage +400 V). b, CE-ESI-MS/MS analysis for $m/z$ 991 representing one repeat of the CPS containing both MeOPN modifications (positive ion mode, orifice voltage +400 V). Pentose is $\beta$-D-Ribf, HexANGro is $\alpha$-D-GlcPAl6NGro, HexNAC is $\beta$-D-GalPNAc, Hep is $\alpha$-D-glycero-$\alpha$-L-gluco-heptopyranose, and MeOPN is O-methyl phosphoramidate.
MeOPN CPS Modification in C. jejuni

### Table 2

| Atom | Type | *a* | *b* | *c* | *d* |
|------|------|-----|-----|-----|-----|
| A1   | CH   | 5.36 | 5.36 | 106.1 | 106.1 |
| A2   | CH   | 4.19 | 4.18 | 81.1  | 81.1  |
| A3   | CH   | 4.32 | 4.33 | 70.7  | 70.8  |
| A4   | CH   | 4.13 | 4.13 | 84.0  | 83.9  |
| A5/5' | CH  | 3.72/3.89 | 3.71/3.89 | 62.9  | 63.1  |
| B1   | CH   | 5.12 | 5.12 | 98.8  | 98.8  |
| B2   | CH   | 3.94 | 3.93 | 73.2  | 73.1  |
| B3   | CH   | 4.08 | 4.08 | 73.2  | 73.2  |
| B4   | CH   | 3.93 | 3.93 | 76.2  | 76.1  |
| B5   | CH   | 4.33 | 4.32 | 72.5  | 72.5  |
| B6   | C    | 171.3 | 171.4 |
| B7   | CH   | 4.05 | 4.05 | 53.9  | 53.9  |
| B8/8a | CH  | 3.66/3.73 | 3.66/3.73 | 61.2  | 61.2  |
| B7/7a | CH  | 3.23/3.54 | 3.88/3.98 | 62.6  | 63.3  |
| C1   | CH   | 5.02 | 5.01 | 104.3 | 104.4 |
| C1'  | CH   | 5.08 | 5.08 | 104.1 | 104.1 |
| C2   | CH   | 4.11 | 4.11 | 62.2  | 62.2  |
| C2'  | CH   | 4.28 | 4.28 | 61.6  | 61.6  |
| C3   | CH   | 4.23 | 4.23 | 73.9  | 73.9  |
| C3'  | CH   | 4.93 | 4.93 | 78.8  | 78.8  |
| C4   | CH   | 4.14 | 4.14 | 82.3  | 82.3  |
| C4'  | CH   | 4.33 | 4.33 | 81.7  | 81.7  |
| C5   | CH   | 3.86 | 3.86 | 78.5  | 78.5  |
| C6/C6' | CH  | 3.75/3.89 | 3.76/3.89 | 61.8  | 61.8  |
| C7   | C    | 175.1 | 175.1 |
| C8   | CH   | 2.05 | 2.05 | 23.0  | 22.9  |
| D1   | CH   | 5.58 | 5.57 | 97.9  | 97.9  |
| D2   | CH   | 3.64 | 3.64 | 71.8  | 71.8  |
| D3   | CH   | 3.64 | 3.64 | 82.2  | 82.2  |
| D4   | CH   | 4.33 | 4.33 | 74.6  | 74.6  |
| D5   | CH   | 4.43 | 4.42 | 70.8  | 70.9  |
| D6   | CH   | 3.78 | 3.78 | 78.8  | 78.2  |
| D7/D7' | CH  | 3.88 | 3.88 | 62.8  | 62.6  |
| D8   | CH   | 3.61 | 3.61 | 60.7  | 60.8  |
| D9   | CH   | 3.55 | 3.54 | 59.3  | 59.3  |
| E    | CH   | 3.77/3.78 | 3.79 | 54.6  | 54.6  |

* Represents residue B that is substituted with N-glycerol.
* Represents residue B that is substituted with ethanolamine. Residue A is β-D-Ribf. B is the amide of α-D-GlcNAc; C is β-D-GalNAc (for 11168H, C and D are β-D-GalNAc without and with the MeOPN modulation, respectively), and D is α-D-gluco-α-L-gluco-heptopyranosyl. Carbon and proton chemical shifts were referenced to an internal acetone standard (δ(C) 22.25 ppm and δ(H) 31.07 ppm). Error for δ(C) is ± 0.02 ppm, for δ(H) is ± 0.2 ppm and for δ(C) is ± 0.2 Hz.

MeOPN is a phase-variable CPS modification in the NCTC11168 strain (13). A 1H-31P HMQC experiment revealed the location of the novel MeOPN group to be at C-4 of α-L-gluco-α-L-gluco-heptopyranosyl (Fig. 4b). This finding is supported by the proton chemical shifts for 11168H CPS sugars that are nearly identical to those reported for the NCTC11168 CPS (8, 13) with the exception of H-4 of residue D that is down-fielded by 0.76 ppm. This downfield shift is consistent with the effects of phosphoramidation reported for H-3 of the β-D-GalNAc CPS sugar in the NCTC11168 strain (0.64 ppm), and H-3 of the β-D-Fru residue in the G1 strain of C. jejuni (0.74 ppm) (8, 13, 14). Resonances observed for N-glycerol, ethanolamine, an OCH₃ group (D9) at C-6 of residue D, and a novel OCH₃ group (D8) that was determined to be located at C-3 of residue D (using heteronuclear multiple-bond correlation and HMCTOXY experiments; data not shown), indicated that the CPS produced by the 11168H strain is structurally heterogeneous. CE-ESI-MS/MS experiments of purified 11168H CPS were used to corroborate NMR findings, and to further characterize the extent of structural heterogeneity (Fig. 5 and Table 3). Fragment ions observed at m/z 1101 and 898 confirmed the location of the novel MeOPN and –OCH₃ group on residue D (Fig. 5a). Ions observed at m/z 283, 415, 549, 634, 664, 867, 884, 947, 977, and 1180 demonstrated that both MeOPN groups can be variably methylated, whereas those observed at m/z 533, 665, and 791 showed that at least one –OCH₃ group is variably present on residue D. Of particular interest, CE-ESI-MS/MS of m/z 991 established that at least some repeating units within the CPS have both MeOPN groups present (Fig. 5b). Based on these results, the CPS produced by C. jejuni 11168H was concluded to be structurally heterogeneous and to have the same repeating unit as the NCTC11168 strain with the addition of a novel –OCH₃ group and MeOPN group at C-3 and C-4 of α-L-gluco-α-L-gluco-heptopyranosyl, respectively (Fig. 1).

Complementation Studies of cj1421 and cj1422—Based on BLAST searches that indicated cj1421 and cj1422 have high sequence similarity with sugar transferases, that cj1421 and cj1422 share long regions of sequence identity (18), and the phenotypes obtained for the cj1421 and cj1422 mutants (Fig. 3, h and i), it seemed likely that these genes encode transferases that add the MeOPN to CPS sugars. To conclusively define the role of cj1421 and cj1422, complementation studies were performed using the 11168H cj1421/cj1422 double mutant background (Fig. 6).

One-dimensional, 31P-decoupled, 1H-31P HSQC HR-MAS NMR analysis of 11168H wild type cells revealed the expected signals for the MeOPN groups at C-3 of residue C (δ(C) 3.77 ppm) and the novel MeOPN at C-4 of residue D (δ(C) 3.79 ppm) (Fig. 6a, note that spectra shown in Fig. 6 were 31P-decoupled to eliminate 31P scalar couplings resulting in the appearance of only one peak for each MeOPN). Mutation of cj1421 resulted in the loss of the MeOPN signal at δ(C) 3.77 ppm which suggested this gene encodes a transferase that adds the MeOPN to residue C (Fig. 6b). To ensure that other CPS structures were not affected by this mutation, the cj1421 CPS was isolated and characterized using identical techniques described for the 11168H wild type CPS (see above). NMR and mass spectrometric analyses of purified cj1421 CPS confirmed that it is structurally...
MeOPN CPS Modification in C. jejuni

TABLE 3

Positive ion CE-ESI-MS data (+400 V orifice voltage), calculated masses, and proposed fragments for CPS isolated from the 111168H strain of C. jejuni

| Molecular mass (m/z) | Structure |
|---------------------|-----------|
| Observed            | Calculated| Difference |
| 112.2               | 112.0     | 0.2        |
| 204.3               | 204.2     | 0.1        |
| 250.2               | 250.2     | 0.0        |
| 283.4               | 283.2     | 0.2        |
| 297.4               | 297.2     | 0.2        |
| 314.2               | 314.2     | 0.0        |
| 336.3               | 336.3     | 0.0        |
| 352.3               | 352.3     | 0.0        |
| 382.3               | 382.3     | 0.0        |
| 415.4               | 415.3     | 0.1        |
| 429.1               | 429.3     | 0.2        |
| 532.2               | 532.4     | 0.2        |
| 533.2               | 533.4     | 0.2        |
| 549.5               | 549.4     | 0.1        |
| 555.6               | 555.5     | 0.1        |
| 563.2               | 563.4     | 0.2        |
| 585.4               | 585.5     | 0.1        |
| 634.2               | 634.5     | 0.3        |
| 664.6               | 664.5     | 0.1        |
| 665.5               | 665.5     | 0.2        |
| 678.4               | 678.5     | 0.1        |
| 695.2               | 695.5     | 0.2        |
| 775.4               | 775.7     | 0.3        |
| 791.4               | 791.7     | 0.3        |
| 805.4               | 805.7     | 0.3        |
| 867.3               | 867.7     | 0.4        |
| 868.7               | 868.7     | 0.0        |
| 884.5               | 884.7     | 0.2        |
| 898.8               | 898.8     | 0.0        |
| 917.4               | 917.7     | 0.3        |
| 957.8               | 957.8     | 0.0        |
| 991.8               | 991.8     | 0.0        |
| 1101.8              | 1101.9    | 0.1        |
| 1180.6              | 1180.9    | 0.3        |
| 1390.0              | 1390.3    | 0.3        |

identical to that produced by the 11168H wild type with the exception of missing the MeOPN at the 3-position of residue C (Fig. 1, Table 2, Supplemental Table 3, and Supplemental Figs. 1 and 2). In contrast, mutation of cj1422 resulted in the loss of the signal for the novel MeOPN group at δH 3.79 ppm that indicated this gene encodes a transferase responsible for adding the MeOPN to C-4 of residue D (Fig. 6c). By comparing the chemical shifts for the anomic protons of cj1422 CPS sugars to those reported for NCTC11168 (13), it was concluded that other CPS structures were not affected by this mutation. Interestingly, mutation of cj1421 and cj1422 resulted in the complete loss of both MeOPN modifications (Fig. 6d). Complementation of cj1421 in cis in the double mutant background resulted in restoration of the MeOPN on residue C (Fig. 3e), whereas complementation of cj1422 in cis was found to restore the MeOPN found on residue D (Fig. 3f). Based on the results of these complementation studies, cj1421 and cj1422 were concluded to encode MeOPN transferases; Cj1421 adds the MeOPN to β-D-GalNAc, whereas Cj1422 adds the MeOPN to α-L-gluco-heptopyranose.

DISCUSSION

Capsular polysaccharides are surface-exposed glycans on the bacterial cell that often contribute to virulence and mediate interactions between the pathogen, host, and the environment. There is interest in these glycans because therapeutic directed toward CPS have been successful in the control of bacterial infections. For example, the Neisseria meningitidis vaccine that is currently in use targets the conserved CPS structure of group C organisms (29). For C. jejuni, however, where at least 60 different serostrains have been identified to date, there does not seem to be a dominant CPS structure. In this study we have established the commonality of the MeOPN CPS modification in C. jejuni, shown that the MeOPN can be used to detect C. jejuni cells from the natural avian host, and have identified several genes implicated in its biosynthesis and transfer.

The majority of the C. jejuni isolates surveyed during this study were found to express the MeOPN CPS modification. This observation points to the commonality of the MeOPN in C. jejuni and is further corroborated by whole genome microarray studies that showed 61 of the 111 C. jejuni strains tested had positive hybridization reactions for genes cj1421 and cj1422 (30). The fact that none of the 18 C. coli strains examined in this study were found to express an MeOPN indicates that the MeOPN is specific for C. jejuni. Furthermore, that the MeOPN was readily detected from the cecal contents of colonized chicks indicates that the MeOPN is expressed by C. jejuni cells inhabiting the avian gastrointestinal tract and thus could potentially be used as a diagnostic marker for C. jejuni colonization. How-
MeOPN CPS Modification in C. jejuni

However, MeOPN expression is not necessary for C. jejuni colonization of chicks because MeOPN mutants in both 11168H and 81-176 backgrounds colonized as well as the wild type (results not shown).

Analysis of a library of CPS mutants resulted in the identification of genes that are directly implicated in the synthesis or transfer of MeOPN. The definitive role of many of these genes remains to be established; however, BLAST searches have provided putative functions (Table 4). For example, CJ1416 shows 32% identity to LicC, a protein found in Neisseria spp., Haemophilus influenzae, and Streptococcus pneumoniae (32). The lic genes are involved in the production of phosphorylcholine, a small phosphorus-containing molecule that decorates surface glycoconjugate structures. LicC is the cytidylyltransferase that activates phosphorylcholine, and thus it is possible that CJ1416 generates a nucleotide-linked MeOPN that is then recognized by the CJ1421 and CJ1422 transferases. Conserved domain searches for CJ1417 identified a type 1 glutamine amidotransferase. Glutamine amidotransferase activity catalyzes the transfer of ammonia from the amide side chain of glutamine to an acceptor substrate. Using isotope-labeled $^{15}$NH$_4$Cl, we previously showed that 11168H is able to incorporate exogenous ammonia into the MeOPN moiety of its CPS (19). One could imagine a role for CJ1417 in transferring ammonia to a phosphorus atom thereby forming the MeOPN. Similarly, the CJ1415 and CJ1418 proteins resemble phosphate kinases and may therefore play a key role in phosphoramide biosynthesis.

CJ1421 and CJ1422 are 55% identical to each other, with strong conservation in the N and C termini (18), which is suggestive of a gene duplication event. Using complementation studies of a double cj1421/cj1422 11168H mutant, we demonstrated that these genes encode MeOPN transferases. In the 11168H strain, CJ1421 adds the MeOPN to $\beta$-d-GalNAc, whereas CJ1422 adds the MeOPN to d-glycero-\alpha-L-gluco-heptopyranose. The different strains that are known to express the MeOPN modification have homologues of these genes within their CPS loci (18). The NCTC12517 strain (MeOPN attached at C-4 of $\beta$-d-GlcNAc) (15) has HS19.07, which is a cj1421 homologue; the G1 strain (MeOPN attached at C-3 of $\beta$-d-Fru sugars) (14) also has a cj1421 homologue, whereas the 81-176 strain (MeOPN attached at C-2 of $\alpha$-d-Galp) (16) has HS23/36.07, which is a cj1422 homologue. CJ1421 and CJ1422, as well as other genes within the CPS biosynthesis loci, were shown to undergo phase variation because of the presence of homopolymeric tracts (21). This phase variability explains much of the structural heterogeneity within the CPS structures produced by C. jejuni and also why the NCTC11168 strain expresses only one MeOPN despite having the genetic potential to express both MeOPN groups.

In this study, we have shown that the second MeOPN group is located at C-4 of d-glycero-\alpha-L-gluco-heptopyranose in the 11168H strain. The MeOPN groups are important sources of structural heterogeneity because they are variably methylated.

![FIGURE 6. Complementation studies for MeOPN transferase genes cj1421 and cj1422. A $^{31}$P-decoupled, $^{1}$H-$^{31}$P HSQC HR-MAS NMR experiment (256 scans, $^{1}$H$_{13}$P = 12 Hz) was used to examine intact cells for the following: a, C. jejuni 11168H wild type; b, cj1421 mutant; c, cj1422 mutant; d, cj1421/cj1422 double mutant; e, cj1421/cj1422 double mutant complemented in cis with cj1421; f, cj1421/cj1422 double mutant complemented in cis with cj1422. For all NMR experiments, 40 $\mu$l of cells were examined at 500 MHz ($^{1}$H) using 256 scans.](image-url)

**TABLE 4**

Summary of proteins identified in this study to be involved in MeOPN biosynthesis and transfer

| Protein            | Genome re-annotation function | CPS phenotype by NMR | Predicted function from this study |
|--------------------|-------------------------------|-----------------------|-----------------------------------|
| CJ1415             | Putative adenylylsulfate kinase (Adenosine 5'-phosphosulfate kinase) | Loss of MeOPN         | Phosphate biosynthesis for MeOPN? |
| CJ1416             | Putative sugar nucleotidyltransferase (Glc-1-P cytidylyltransferase, LicC) | Loss of MeOPN         | Nucleotidyltransferase to generate activated NDP-MeOPN? |
| CJ1417             | Putative amidotransferase (Type 1 glutamine amidotransferase-GATase1) | Loss of MeOPN         | Amidation of phosphate? |
| CJ1418             | Putative transferase (pyruvate phosphate dikinase) | Loss of MeOPN         | Phosphate biosynthesis for MeOPN? |
| CJ1421             | Putative glycosyltransferase | Loss of MeOPN on GalNAc | MeOPN transferase to GalNAc |
| CJ1422             | Putative glycosyltransferase | Loss of MeOPN on Hep   | MeOPN transferase to Hep |

* GATase indicates glutamine amidotransferase.
and because one or two MeOPN groups can be present within one repeat of the CPS. Based on the signals originating from the MeOPN groups on the cell surface that appeared in a 1:1 ratio (Fig. 2a), it can be concluded that there is an equal number of each MeOPN group within the 11168H CPS. In light of the variably methylated MeOPNs on β-D-GalNAc and/or β-D-glycerol-α-L-glucopyranosyl, variable N-glycerol or ethanolamine groups on α-D-GlcPA, and two variable –OCH3 groups on β-glycerol-α-L-glucopyranosyl, the 11168H strain produces the most decorated and structurally heterogeneous CPS reported for C. jejuni. Using the visual molecular dynamics software (33), we constructed a model for the repeating unit of the CPS produced by C. jejuni 11168H. The CPS structure consists of β-D-Ribp (A), α-D-GlcPA6(NGro) (B), β-D-GalNAc (C), and 3,6-di-O-methyl-β-D-glycerol-α-L-glucopyranosyl (D). As can be seen, the variable CPS modifications (darker color) such as MeOPNs, –OCH3(OMe), N-glycerol(NGro), or ethanolamine (not shown) groups that decorate the repeating unit (lighter colors) would be prominent structural features on the cell surface for this strain. OH groups have been removed to simplify the appearance of the model.

Because the MeOPN is the only CPS structure known to be conserved among C. jejuni strains, it has potential use as a diagnostic marker and vaccine candidate for this bacterium. Several lines of evidence now indicate an important biological role for the MeOPN in C. jejuni; it is a prominent structural feature on the cell surface of multiple strains; it is expressed by cells inhab-

iting the natural avian host; and several genes are dedicated to its biosynthesis/transfer. A link between MeOPN expression and pathogenicity cannot be eliminated given its high prevalence among enteritis, Guillain Barré syndrome, and Miller-Fisher syndrome strains. Future work will focus on elucidating the biological relevance of the MeOPN group, functional characterization of the enzymes involved in MeOPN biosynthesis, and exploitation of this common structure in C. jejuni.

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MeOPN CPS Modification in C. jejuni

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