The Polysaccharide Capsule of Campylobacter jejuni Modulates the Host Immune Response

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The polysaccharide capsule of *Campylobacter jejuni* 81-176 modulates the host immune response

Alexander C. Maue¹, Krystle L. Mohawk¹, David K. Giles², Frédéric Poly¹, Cheryl P. Ewing¹, Yuening Jiao³, Ginyoung Lee³, Zuchao Ma³, Mario A. Monteiro³, Christina L. Hill¹, Jason S. Ferderber¹, Chad K. Porter¹, M. Stephen Trent⁴, and Patricia Guerry¹#

¹Enteric Diseases Dept., Naval Medical Research Center, Silver Spring, MD
²Dept. of Biological and Environmental Sciences, University of Tennessee at Chattanooga, TN
³Dept. of Chemistry, University of Guelph, Guelph, Ontario
⁴The Section of Molecular Genetics and Microbiology, University of Texas at Austin, Austin, TX

#Corresponding author. Mailing address: Enteric Diseases Dept., Naval Medical Research Center, 503 Robert Grant Ave, Silver Spring, MD 20910; Telephone: 301-319-7662; Email: patricia.guerry@med.navy.mil

Running title: Immune modulation by *C. jejuni* capsule
Abstract

*Campylobacter jejuni* is a major cause of bacterial diarrheal disease worldwide. The organism is characterized by a diversity of polysaccharide structures, including a polysaccharide capsule. Most *C. jejuni* capsules are known to be decorated non-stoichiometrically with methyl phosphoramidate (MeOPN). The capsule of *C. jejuni* 81-176 has been shown to be required for serum resistance, but here we show that an encapsulated mutant lacking the MeOPN modification, *mpnC*, was equally sensitive to serum killing as the non-encapsulated mutant. A non-encapsulated mutant, *kpsM*, exhibited significantly reduced colonization compared to wildtype 81-176 in a mouse intestinal colonization model, and the *mpnC* mutant showed an intermediate level of colonization. Both mutants were associated with higher levels of IL-17 expression from lamina propria CD4+ cells compared to cells from animals infected with 81-176. In addition, reduced levels of TLR-4 and -2 activation were observed following in vitro stimulation of human reporter cell lines with *kpsM* and *mpnC* compared to wildtype 81-176. The data suggest that the capsule polysaccharide of *C. jejuni*, and the MeOPN modification, modulate the host immune response.
Introduction

*Campylobacter jejuni* is one of the major causes of bacterial diarrhea worldwide. The organism is unusual among enteric pathogens in that it expresses a polysaccharide capsule (CPS) that contributes to serum resistance, invasion of intestinal epithelial cells in vitro and virulence in ferret and *Galleria mellonella* larvae models of disease (3, 8, 46). CPS is the major serodeterminant of the Penner serotyping scheme of *C. jejuni* (27) of which there are 47 serotypes, a reflection of the diversity of polysaccharide capsular structures in *C. jejuni*. In addition to variation in sugar composition, the CPS can be modified with ethanolamine, glycerol and O-methyl phosphoramidate (MeOPN). The MeOPN modification, which is found on about 75% of *C. jejuni* CPSs, has been shown to modulate cytokine release from mouse dendritic cells and to be a key determinant in virulence in the moth larvae model of disease (8). Both CPS expression itself (3) and expression of the modifications are phase variable due to slip strand mismatch repair (20, 28, 40). Thus, reversible phase variations in multiple genes result in mixed populations of wildtype cells, some of which express CPS and others that do not (3). Similarly, the levels of the MeOPN modifications found on the CPS are present in non-stoichiometric amounts because of phase variation in genes encoding the enzymes involved in transfer of these groups to specific sugars (37).

We have shown that a polysaccharide conjugate vaccine composed of the capsule of strain 81-176 conjugated to carrier protein CRM197 showed significant protection against diarrheal disease in a non-human primate model of
diarrhea, also suggesting a role for CPS in virulence (38). Here we further demonstrate that the polysaccharide CPS and the MeOPN modification both play significant roles in modulation of several aspects of the immune response, including serum resistance, activation of NF-κB, and cytokine induction in vivo.

Materials and Methods

Bacterial strains and media. C. jejuni strain 81-176, its motile, isogenic kpsM and the complement of that mutant have been described (3). Bacteria were routinely cultivated microaerobically on Mueller Hinton (MH) agar supplemented with antibiotics as appropriate. For serum resistance assays, strains were grown in biphasic MH cultures for 18-20 h at 37°C. For mouse infection studies, strains were inoculated in MH broth to an OD₆₀₀ of ~0.01-0.05 and incubated with shaking in microaerobic conditions at 37°C for 18 hours.

Mutation and complementation of a gene for biosynthesis of MeOPN in C. jejuni 81-176. A region of the CPS locus of the 81-176 chromosome encoding genes for MeOPN synthesis (37) was cloned as a PCR fragment into BamHI-digested pBluescript. The primers used were pg08.90 (CGGGATCCGGAATGCTGTATATAGGAGTTGGA) in CJJ81176_1417 (labeled mpnA in Fig. 1) and pg08.91 (CGGGATCCCATCGAAGCATCATCTTCAACTTGAGT) in CJJ81176_1413 (kpsC). Both primers introduced a BamHI site at the 5’ ends, as indicated by the underlining. The resulting plasmid was subjected to transposon mutagenesis using an in vitro Tn5-based transposition system (Epicentre, Madison, WI) with a
chloramphenicol resistance (Cm<sup>+</sup>; cat) cassette, and the insertion points were identified by sequencing individual insertions with primers within the cassette, as previously described (11, 19, 26). A clone with a non-polar insertion into gene CJJ81176_1415 (mpnC in Fig. 1) was identified; the insertion was 472 bp into the 762 bp gene. This plasmid was used to electroporate C. jejuni 81-176 to Cm<sup>+</sup>, and the resulting mutant was confirmed to have undergone a double crossover by PCR with primers that bracketed the insertion point of the transposon. The mutant was complemented by cloning a wildtype allele of mpnC into pRY107/28, which is pRY107 (56) containing the σ<sup>28</sup> promoter of flaA cloned between the XbaI and BamHI sites. The wildtype mpnC gene was PCR amplified using high fidelity polymerase (Clontech) and primers pg08.155 (5'-CGGGATCCGTATAATGTGGCATATTGAAAGAG-3') and pg08.150 (5'-CCGCTCGAGCTCTTAAACTCATCTCCATCGAGATAAATAAG-3') that introduced BamHI and XhoI sites, respectively. This fragment was cloned into BamHI-XhoI digested pRY107/28. The plasmid complement was introduced into 81-176 mpnC by conjugal transfer from E. coli DH5α containing RK212.2, as previously described, with selection on kanamycin (18).

**31P-Nuclear Magnetic Resonance (NMR) spectrometry.** Preparations containing the CPSs were dissolved in D<sub>2</sub>O and 31P NMR was performed on a 400 MHz Bruker NMR instrument. Ortho-phosphoric acid was used as the external reference (δ 0.00).

**Serum survival assays.** 18 h cultures of C. jejuni grown in MH biphasic media were washed and adjusted to an OD<sub>600</sub> 0.1 in MEM medium. Aliquots
(100 ul) were added to wells of a 24-well plate containing 900 ul of pre-warmed MEM media supplemented with 10% normal human serum (Sigma; NHS), and incubated under microaerobic conditions at 37°C. The percentage of survivors was determined by serial dilution onto MH agar plates. Assays were run in duplicate 3-4 times.

**Mouse infection experiments.** Seven- to eight-week old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were housed in groups of 10 with access to food and water ad libitum. For infection with *C. jejuni*, one liter of broth culture was harvested by centrifugation and resuspended in PBS. The inocula were normalized by OD600 to ~10^{11} CFU/mL, and animals were inoculated intragastrically with 100 μL of the cell suspension. The inocula doses were validated on MH agar plates prior to and immediately after infection of the animals.

**Assessment of colonization.** Fecal collections were performed by allowing individual mice to defecate in clean, empty shoebox cages prior to returning to group housing. Feces were collected using forceps into 5 mL Falcon snap-cap tubes and then diluted 1:10 by weight into PBS. Various stool dilutions were plated onto campylobacter selective media (CVA plates; Remel) and incubated under microaerobic conditions at 42°C for 2 days.

**Lymphocyte isolation.** At indicated time points following oral challenge, mice were sacrificed and small intestines were removed to recover lymphocytes from the lamina propria (LPL; lamina propria lymphocytes) as described.
previously (32) with some modifications. In brief, Peyer’s patches (PP) were removed from the intestines and intestines were cleared of contents using forceps, opened longitudinally and then cut into ~5mm sections. Intraepithelial lymphocytes (IEL) were removed from these intestinal sections by placing the tissue in a solution of 1 mM DTT/1 mM EDTA at 37°C for two 20 minute incubations. After each incubation, the supernatant was removed and replaced with fresh DTT/EDTA. To isolate LPLs, the remaining intestinal pieces were digested with collagenase D (Roche, 1 mg/ml) and DNase I (Sigma, 40 µg/ml) for two 1 h incubations at 37°C. The supernatant was removed following each incubation and replaced with fresh media. Following the digestion of small intestinal tissue sections, cells were pelleted by centrifugation and LPLs were isolated using a discontinuous (80-40%) Percoll gradient.

**Intracellular cytokine staining.** LPL were cultured *in vitro* for 4-6 hours in the presence of media alone or phorbol-12-myristate-13-acetate (PMA) (Sigma, 20 ng/ml) and ionomycin (Sigma, 0.5 µg/ml). Media was DMEM supplemented with 10% FBS, 2mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, non-essential amino acids, 50 µM 2-mercaptoethanol, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Protein transport was inhibited with the addition of brefeldin A (10 µg/ml). Following culture, LPLs were stained with CD4 APC. Cells were fixed in 4% formaldehyde prior to permeabilization with 0.1% saponin (in PBS and 1% FBS). Intracellular staining was performed using anti-mouse IFN-γ FITC (eBioscience) or anti-mouse IL-17 PE (eBioscience). Cells were then washed and resuspended in 1% formaldehyde prior to analysis on a Becton
Dickinson FACScan equipped with red and blue lasers (i.e. 5-color capability).

Data were analyzed using FlowJo software (TreeStar).

**TLR signaling assay using whole bacteria.** The following cell lines were purchased from InvivoGen: HEK-Blue-hTLR4, HEK-Blue-hTLR2 and THP1-XBlueTM-MD2-CD14. The human epithelial kidney (HEK) 293 cells are stably transfected with either human TLR4, MD2 and CD14 (HEK-Blue-hTLR4) or human TLR2 and CD14 (HEK-Blue-hTLR2). THP1-XBlueTM-MD2-CD14 cells are derived from the human monocytic THP-1 cell line and are stably transfected with MD2 and CD14. These HEK-Blue and THP1-XBlue clones also stably express secreted embryonic alkaline phosphatase (SEAP) under the control of a promoter inducible by NF-κB and activator protein 1 (AP-1). Thus, stimulation of toll-like receptors will result in an amount of extracellular SEAP in the supernatant that is proportional to the level of NF-κB induction. The HEK cell lines were maintained in standard DMEM with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) supplemented with 4.5 g/L glucose, 2mM L-glutamine, 50 U/mL penicillin, 50ug/ml streptomycin, 100ug/ml Normocin (InvivoGen) and 1X HEK-Blue selection (InvivoGen) in a 5% saturated CO₂ atmosphere at 37°C.

The THP1 cell line was maintained in standard RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) supplemented with 4.5 g/L glucose, 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 10mM HEPES, 1mM sodium pyruvate, 50 U/mL penicillin, 50ug/ml streptomycin, 100ug/ml Normocin (InvivoGen), 200ug/ml Zeocin (InvivoGen) and 250 ug/ml G418 (InvivoGen) in a 5% saturated CO₂ atmosphere at 37°C.
The induction of TLR signaling in HEK-Blue-hTLR4, HEK-Blue-hTLR2 and THP1-XBlueTM-MD2-CD14 clones was assessed by measuring SEAP activity using QUANTI-Blue™ colorimetric assay (InvivoGen). The assays were performed according to manufacturer’s protocols. Briefly, cells were seeded in a 96-well plate in triplicate (2.5 X 10⁴ cells/well for HEK-Blue-hTLR4, 5 X 10⁴ cells/well for HEK-Blue-hTLR2 and 1 X 10⁵ cells/well for THP1-XBlueTM-MD2-CD14). Whole bacterial cells were grown on Mueller Hinton agar for 16 h, collected from the plate, washed and resuspended in sterile PBS. 10-fold serial dilutions were prepared based on OD₆₀₀ to yield the number of bacteria inoculated into each well. CFU were confirmed by plating serial dilutions on MH agar. Erythromycin (50 ug/ml) was included to prevent bacterial growth during incubation, and other antibiotics used in the media for cell propagation were omitted in the assay. After 18 h incubation, supernatants (20ul) were transferred to a 96-well plate and incubated at 37°C with QUANTI-Blue (180ul). SEAP activity was measured by reading OD₆₅₅ nm with a Synergy Mx multi-mode microplate reader (BioTek).

**Statistical analyses.** Differences in mouse colonization level, as assessed by the number of organisms shed (log 10 of CFU/gram feces), were compared using a repeated measures analysis of variance with the C. jejuni strain as the between animal factor (i.e., wild type, kpsM mutant, mpnC mutant) and collection time-points as the repeated factor. The covariance structure was modeled using a first-order antedependence model. A Tukey adjustment was utilized to control the type I error rate. Comparisons of the proportion of mice
infected by strains over time were made using a cox proportional hazards model. These analyses were conducted with SAS version 9.2 for Windows (SAS Institute, Inc., Cary, North Carolina) using a two-tailed alpha of 0.05.

Statistical analyses of complement killing, intracellular cytokine expression, and TLR assays were analyzed using student's t test. Differences were considered significant at $P < 0.05$.

Results

Construction of a mutant in the MeOPN biosynthetic pathway of 81-176. McNally et al. (37) identified the genes in *C. jejuni* strain NCTC 11168 (CJ1415-18) that were responsible for MeOPN synthesis, as well as two distinct MeOPN transferases that were responsible for attachment of MeOPN to two different sites in the polysaccharide CPS of this strain. CJ1415-CJ1418 are highly conserved among *C. jejuni* strains, while the transferases are more variable based on differences in attachment of the MeOPN to sugars. The genes corresponding to CJ1415-1418 in 81-176 are CJJ81176_1414-CJJ81176_1417. Since the function of genes has been established in NCTC 11168, we have named the genes for MeOPN synthesis *mpnA-D*, shown in Fig. 1A, for clarity in discussing these conserved genes in different strains. A mutant in *mpnC* in 81-176 was shown to lack MeOPN by $^{31}$P-NMR, as predicted based on the NCTC 11168 data (37), and MeOPN was restored when the mutant was complemented (Fig. 1). The *mpnC* mutant produced CPS as determined by both NMR and
MeOPN contributes to serum resistance. There have been several reports demonstrating that non-encapsulated mutants of C. jejuni are more sensitive to normal human serum than wildtype strains (3, 29). Comparable data to those published for 81-176 and its isogenic kpsM mutant (3) are shown in Fig. 2. Surprisingly, the mpnC mutant, expressing the polysaccharide CPS lacking MeOPN, displayed the same pattern of serum killing as the kpsM mutant lacking all CPS (Fig. 2). The mpnC mutant was significantly more sensitive than wildtype to complement at both 60 min (P <0.001) and 120 min (P<0.005). When the mpnC mutant was complemented in trans, serum resistance returned to levels comparable to wildtype (Fig. 2).

Capsule is required for prolonged mouse colonization. The ability of the kpsM and mpnC mutants to colonize mice was compared to wildtype in a series of experiments. Animals were intragastrically infected with C. jejuni and colonization was monitored post-infection by fecal shedding. Following infection, wildtype C. jejuni 81-176 colonized mice on average at levels exceeding 10^6 CFU/g feces (Fig. 3A). This high level of colonization was maintained for greater than 15 days before counts began to drop below the initial colonization levels.

Mice infected with the kpsM mutant generally had early colonization levels similar to that of wildtype (Fig. 3A). In addition, the kpsM-infected mice had a shorter duration of colonization compared to those infected with wildtype (P=0.06). The majority of kpsM-infected mice cleared the infection by day 18 in comparison to
the wildtype-infected mice that remained colonized at some level through day 28 post-infection (the last day tested) (Fig. 3A). Thus, despite the similar level of colonization seen early post-infection, by day 14 there was a statistically significant difference in colonization levels of mice infected with *kpsM* when compared to wildtype (P <0.01).

In parallel experiments, colonization capacity of the *mpnC* mutant was compared to wildtype. Upon infection with the *mpnC* mutant, mice shed similar numbers of *C. jejuni* in their stool in comparison to wildtype (Fig. 3B). In fact, there were no significant differences in stool counts between the *mpnC* mutant and wildtype during the first 10 days post-infection, and only later in infection did the *mpnC* mutant demonstrate a significant reduction in the level of colonization when compared to wildtype (Fig. 3B; P=0.02 for day 20). Thus, although not directly compared, the colonization ability of the *mpnC* mutant appeared intermediate in nature compared to wildtype and *kpsM* (Fig. 3).

**IL-17 expression from intestinal T cells is modulated by the polysaccharide CPS.** To determine if CPS had a role on immune responses in vivo, mice were orally infected with wildtype 81-176 and *mpnC* or *kpsM* strains. At selected times post-infection, T cells were isolated from small intestine Peyer’s patches, epithelium and lamina propria. Following an ex vivo restimulation, the expression of IL-17 and IFN-γ were determined using intracellular cytokine staining and flow cytometry. Figure 4A shows representative histograms for CD4 LPLs and dot plots demonstrating gating strategies for intracellular cytokine staining (Fig. 4B). Following infection with either *kpsM* or wildtype strains, CD4
cells from the lamina propria of kpsM-infected mice exhibited significantly higher percentages (P < 0.05) of CD4+ cells that expressed IL-17 at day 7 and day 21 (Fig 4C). No difference was observed in IFN-γ expression of CD4+ LPLs isolated from kpsM or wildtype infected mice at either timepoint. Differences in cytokine expression were not observed from PP cells or IELs (data not shown).

Next, the MeOPN modification on CPS was examined for its role in modulating immune responses in vivo. CD4+ LPL from mpnC-infected mice did not express significantly higher percentages of IL-17+ cells on day 7 compared to LPL isolated from mice orally challenged with wildtype 81-176 (Fig 4D). However, on day 21 post-infection, mice colonized by mpnC exhibited significantly higher percentages (P < 0.01) of CD4+ LPLs expressing IL-17 than animals infected with wildtype. In addition, no significant differences were seen in other lymphocyte subsets or in IFN-γ expression patterns (data not shown).

**Effects of CPS on TLR-signalling.** To determine the impact of CPS production and modification on TLR activation, we performed reporter cell signaling assays with whole bacteria. The kpsM mutant exhibited significantly higher activation than wildtype from $10^4$ to $10^7$ CFU for hTLR4 activation and from $10^5$-$10^8$ CFU for hTLR2 activation (Fig. 5). Although similar results were seen with the mpnC mutant, the lack of complete complementation confounds these results (data not shown).

We subsequently compared the overall TLR-receptor activation of kpsM and mpnC mutants using a human monocytic reporter line that expresses several TLRs, including TLR1, TLR2, TLR4, TLR6, TLR8, NOD1 and NOD2. Significant
increases (P <0.005) in signaling were observed for both mutant strains compared to wildtype and their complements (Fig. 6). For the kpsM mutant these differences were observed from $10^4$-10$^7$ CFU and for the mpnC mutant the differences were observed between $10^5$-10$^7$ CFU.

Discussion

C. jejuni remains a poorly understood pathogen, in part because of the absence of small animal models that mimic human disease. Following orogastric infection with C. jejuni, adult, immunocompetent mice can become colonized for variable lengths of time, but without the disease symptoms seen in immunodeficient mice (5, 6, 12, 23, 35, 39). Despite the lack of disease, the mouse model can provide information on traits required for colonization, the first step in pathogenesis (39). Here we show that wildtype 81-176 colonizes BALB/c mice better than either an isogenic mutant lacking capsule (kpsM) or a mutant expressing CPS without MeOPN (mpnC). Interestingly, a reduction in colonization ability of a MeOPN-mutant of 81-176 (in the gene corresponding to mpnA) compared to wildtype 81-176 was also reported in MyD88-defective mice (54). In our studies BALB/c mice that were colonized with wildtype 81-176 remained colonized for the duration of the experiments (>21 days). The kpsM mutant showed similar colonization levels for about 9 days before colonization levels dropped. The mpnC mutant colonized at levels that were generally lower than wildtype, although reaching statistical significance only at day 20. Thus,
expression of CPS by *C. jejuni* facilitated colonization in the mouse model. CPS has also been shown to play a role in *C. jejuni* colonization of chickens (2, 16).

Following restimulation, IL-17 production by CD4+ LPLs was reduced in mice colonized by *C. jejuni* 81-176 compared to both mutant strains. Mice colonized by either the *kpsM* or *mpnC* mutants possessed higher levels of IL-17+ CD4+ cells in the small intestine compared to wildtype at day 21, and this increased IL-17 production was associated with a reduction in colonization levels. However, despite the fact that both the *kpsM* mutant and the *mpnC* mutant were associated with higher frequencies of IL-17-producing CD4+ cells in the small intestine compared to wildtype 81-176, the *kpsM* mutant appeared to show a greater reduction in colonization capacity than the *mpnC* mutant suggesting that the presence of the polysaccharide CPS, even without the MeOPN modification, affords some protection against the immune response in the intestine. Although, in vivo cytokine responses were not measured directly in this study, the data suggest that CPS expression, and more specifically, the MeOPN modification on the wildtype capsule, may affect the generation of IL-17 responses in the gut mucosa. Future studies are needed to determine the specificity of the IL-17 response against *C. jejuni* in the intestine.

T helper-17 (Th17) responses have come into focus due to their roles in maintaining intestinal homeostasis (24, 50) and protective immune responses against enteric pathogens (15, 36, 49, 50). The gut Th17 response is composed of both innate and adaptive immune system components. Innate Th17 (iTh17) responses are induced by segmented filamentous bacteria (SFB) that colonize...
the gut (13, 25) and maintain a symbiotic balance between the microbiota and host (50). Specific animal vendors supply mice that are either colonized with SFB or not (Jackson Laboratories, SFB⁺; Taconic SFB⁺) (25). These models can be exploited to evaluate innate or adaptive Th17 responses. Whereas iTh17 responses can be induced relatively quickly by cytokine signals (15, 36), adaptive Th17 responses occur later (days to weeks) (47) and are antigen-specific responses. In our present study, the Th17 responses likely represent an adaptive immune response since Jackson Laboratory mice were used. Key to Th17 responses are the cytokines interleukin-17 (IL-17) and IL-22 (29, 36, 39, 47), and the upstream cytokines that lead to their expression such as IL-1, IL-6, and IL-23 (31, 33, 47). IL-17 is primarily thought to be effective against extracellular pathogens by inducing inflammation and recruiting neutrophils to sites of infection (reviewed in (31). IL-22 exerts its protective effects by inducing epithelial cells to produce anti-bacterial molecules (57). Recently, Th17 responses have been demonstrated to have protective roles against *Salmonella* and *Citrobacter* infections in mice (15, 36). To date only limited data exist regarding Th17 responses and *Campylobacter* infection. Edwards et al. (10) showed that cytokines involved in Th17 responses were induced in colon biopsy tissues following co-culture with *C. jejuni* and that the addition of exogenous IL-17 reduced *C. jejuni* invasion into an intestinal epithelial cell line. However, additional work must be performed to determine the precise role of Th17 immune responses to *C. jejuni.*
Consistent with these data, we have also shown that the presence of the CPS on wildtype 81-176 resulted in reduced activation of both TLR2 and TLR4 using HEK cells expressing each receptor. Our data are consistent with those of Rose et al. (46) who showed that mutants of NCTC 11168 lacking CPS or MeOPN induced higher levels of IL-6, TNF$\alpha$ and IL-10 from mouse dendritic cells compared to the wildtype strain. Using dendritic cells from TLR4$^{-/-}$ mice, they also showed that some of these differences in cytokines were due to TLR4 signalling. Similar down-regulation of the immune response has been observed for other bacterial capsules (9, 42-44, 48, 51). In some cases this inhibition may be due to shielding of the bacterial surface by the capsule and prevention of TLR stimulation. However, the CPS of Neisseria meningitidis actively inhibits TLR2 activation by binding CD14 (30). The MeOPN modification on two distinct CPS structures of C. jejuni has now been shown to modulate cytokine responses and TLR signaling (46), suggesting an active role for this unusual structure. Similarly, the C. jejuni CPS may inhibit binding of complement activators and components to the surface of the bacterial cell, but the fact that the mpnC mutant was as sensitive as the kpsM mutant to complement killing, also suggests an active role for the MeOPN group. In contrast to C. jejuni, modification of Hemophilus influenzae lipopolysaccharide with phosphorycholine, which is also under phase variable expression, enhances sensitivity to complement killing (55). The mechanism by which MeOPN interacts with components of the complement cascade is under investigation.
Collectively, these data indicate the polysaccharide CPS of 81-176, and the MeOPN modification, modulate the immune response to this pathogen and are consistent with previous observations suggesting a stealth strategy by which C. jejuni may avoid the immune response. It has been known for some time that C. jejuni flagellin is unable to induce TLR5 because of structural changes to the monomeric subunit protein that are reflected in changes in filament formation (1, 14). C. jejuni also expresses altered linkages of hydroxyacyl chains on lipid A that reduce TLR4 activation (52), and there is evidence that the N-linked glycan on proteins and certain LOS glycoforms can down regulate IL-6 induction (53). Previous work has shown that the CPS of NCTC 11168, and specifically the MeOPN modification on this CPS, reduced cytokine production from mouse dendritic cells in culture (46). Here, we have demonstrated that a second C. jejuni CPS and the MeOPN modification modulates the immune response at multiple levels, including resistance to complement killing and cytokine induction via NF-κB signalling. The ability to avoid the immune response of the host provides an advantage in establishing colonization by C. jejuni, be it as a commensal in animals or as a pathogen in humans. Moreover, asymptomatic infection by C. jejuni is common among children in the developing world and acute infections are frequently followed by periods of asymptomatic shedding (7, 41, 45), which may be due, at least in part, to the ability of this pathogen to avoid the host immune response. Similarly, recrudescence of infection following appropriate antibiotic treatment in an immunocompetent adult has been reported (4).
One of the hallmark characteristics of *C. jejuni* is its ability to undergo phase variation of surface antigens by slip strand mismatch repair (3, 17, 21, 22, 34, 40). In terms of the polysaccharide CPS, this phase variation occurs at two levels. One is the high frequency on/off reversible expression that was originally described in strain 81-176, such that a culture grown in vitro is a mixed population of encapsulated and unencapsulated variants (3). The other level of phase variation affects CPS structure and is best understood in terms of the MeOPN modification. Thus, all MeOPN transferases that have been described to date are subject to phase variation at homopolymeric tracts of bases, resulting in non-stoichiometric amounts of this modification. The reason for this variability in both CPS expression and structure is not understood, but the data presented here suggest that the polysaccharide CPS, with and without MeOPN, modulates the host immune response at multiple levels. Since *C. jejuni* produces an inflammatory diarrhea, phase variation during replication in vivo may also modulate the severity of illness, and, at least in part, explain variability in severity of symptoms seen with this pathogen.

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Song, X., S. Zhu, P. Shi, Y. Liu, Y. Shi, S. D. Levin and Y. Qian. 2011. IL017RE is the functional receptor for IL-17C and mediates mucosal...


Figure legends

Fig. 1. A. Cartoon of the capsule locus of 81-176. The locus is organized, like other class 2 capsule loci, into conserved regions 1 and 3 encoding proteins involved in capsule assembly and transport and the variable region 2 encoding proteins involved in polysaccharide synthesis. The MeOPN biosynthesis genes (mpnA-D) are found within region 2, but are highly conserved among strains if present and correspond to CJ1415-1418 in NCTC 11168 (37). Genes corresponding to CJJ81176_1418 and CJJ81176_1419 are also highly conserved among strains expressing MeOPN, but mutational analyses have failed to demonstrate a role for these genes in MeOPN synthesis (32). CJJ81176_1420 is annotated as a putative MeOPN transferase. There are 15 additional genes within region 2 of 81-176. B. $^{31}$P-NMR of CPS from wildtype 81-176 (blue), the mpnC mutant (green), and the mpnC mutant complemented in trans (red).

Fig. 2. Sensitivity of 81-176 and mutants to complement killing by NHS. The % survivors are shown after 60 and 120 minutes incubation with 10% NHS. The colors represent: black, 81-176; red, kpsM; green, mpnC; blue, mpnC complemented in trans.

Fig. 3. Colonization of BALB/c mice by C. jejuni strain 81-176 and various CPS mutants. The log CFU/g feces shed by wildtype and either a kpsM mutant (A) or an mpnC mutant (B) are shown over the course of infection. Groups of 10 mice were intragastrically infected with $\sim10^{10}$ CFU. Each data point represents
an individual mouse infected with wildtype (squares) or mutant (circles), and the 
group mean is displayed as a connected line (black for wildtype and dotted for 
mutants). The limit of detection was $10^2$ CFU/g feces. These data are 
representative of 3-4 independent experiments.

**Fig. 4.** IL-17 expression is reduced in small intestinal CD4+ LPLs from mice 
infected with wildtype *C. jejuni* 81-176. BALB/c mice (4-5/group) were orally 
infected with $\sim10^{10}$ CFU *C. jejuni*. At days 7 and 21 post-infection, small 
intestines were removed and processed to isolate LPLs. LPLs were restimulated 
in vitro with PMA (20 ng/ml) and ionomycin (500 ng/ml) for 4-6 h. Protein 
transport was inhibited by addition of Brefeldin A (10 µg/ml). Intracellular 
cytokine staining for IL-17 and IFN-γ was performed on cells and analyzed by 
flow cytometry. (A) Representative histograms demonstrating the percentage of 
CD4+ and CD8+ cells isolated from mouse small intestines. (B) Representative 
dot plots demonstrating intracellular staining for IL-17 and IFN-γ in unstimulated 
and stimulated CD4+ LPLs. (C) Percent expression of IL-17 in CD4+ LPLs from 
mice infected with *kpsM* or wildtype *C. jejuni*. (D) IL-17 expression in CD4+ LPLs 
from mice infected with *mpnC* or wildtype *C. jejuni*. Data represent the mean ± 
SEM. * indicates P < 0.05 and ** indicates P < 0.01 using student’s t test. Data 
are representative of 2-3 independent experiments.

**Fig. 5.** Activation of (A) TLR4 in HEK-293 cells transfected with human 
TLR4-MD2-CD14 and (B) TLR2 in HEK-293 cells stably transfected with 
human TLR2-CD14. TLR activation was monitored colorimetrically using a
SEAP reporter gene placed under the control of an NR-κB inducible promoter.

Ten-fold serial dilutions of whole bacterial cells of the indicated strains of *C. jejuni* were added to each well in triplicate. Values represent the means and standard deviations of one experiment assayed in triplicate. The figure is representative of three independent experiments. Asterisks indicate *P* value <0.005 compared to wildtype 81-176 or the complement. Black lines, wildtype 81-176; red lines, the isogenic *kpsM* mutant; and blue lines, the *kpsM* mutant complemented in trans.

**Fig. 6. Activation of toll-like receptors using a human monocytic (THP-1) reporter cell line by *kpsM* (A) and *mpnC* (B) mutants compared to wildtype 81-176 and complements of each mutant.** Bacteria were added to cells as described in the legend to Fig. 5. Values represent the means and standard deviations of one experiment assayed in triplicate. The figure is representative of three independent experiments. Asterisks indicate *P* value <0.005 compared to wildtype 81-176 or the complement. Black lines, wildtype 81-176; red line, the isogenic *kpsM* mutant; green line, the isogenic *mpnC* mutant; blue lines, the complement of each mutant.