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The Polysaccharide Capsule of *Campylobacter jejuni* Modulates the Host Immune Response

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***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 nonstoichiometrically 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, an *mpnC* mutant, was equally as sensitive to serum killing as the nonencapsulated mutant. A nonencapsulated mutant, a *kpsM* mutant, exhibited significantly reduced colonization compared to that of wild-type 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 interleukin 17 (IL-17) expression from lamina propria CD4$^+$ cells than from cells from animals infected with 81-176. In addition, reduced levels of Toll-like receptor 4 (TLR4) and TLR2 activation were observed following *in vitro* stimulation of human reporter cell lines with the *kpsM* and *mpnC* mutants compared to those with wild-type 81-176. The data suggest that the capsule polysaccharide of *C. jejuni* and the MeOPN modification modulate the host immune response.**

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 (1–3). CPS is the major serodeterminant of the Penner serotyping scheme of *C. jejuni* (4), in 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 larva model of disease (2, 3). Both CPS expression itself (1) and expression of the modifications are phase variable due to slip strand mismatch repair (5–7). Thus, reversible phase variations in multiple genes result in mixed populations of wild-type cells, some of which express CPS and others that do not (1). Similarly, the levels of the MeOPN modifications found on the CPS are present in nonstoichiometric amounts because of phase variation in genes encoding the enzymes involved in transfer of these groups to specific sugars (8).

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 nonhuman primate model of diarrhea, also suggesting a role for CPS in virulence (9). Here, we further demonstrate that the 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* mutant, and the complement of that mutant have been described previously (1). 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 to 20 h at 37°C. For mouse infection studies, strains were inoculated in MH broth to an optical density at 600 nm (OD_{600}) of ~0.01 to 0.05 and incubated with shaking under microaerobic conditions at 37°C for 18 h.

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 carrying genes for MeOPN synthesis (8) was cloned as a PCR fragment into BamHI-digested pBluescript. The primers used were pg08.90 (CGGGATCCGGAATGCCTGCTGTTATAGGAGTTGGA) in CJJ81176_1417 (labeled *mpnA* in Fig. 1) and pg08.91 (CGGGATCCCAT CGAAGCATCATCTTCAACTTGAGC) in CJJ81176_1413 (*kpsC*). Both primers introduced a BamHI site at the 5′ ends, indicated by the underlining. The resulting plasmid was subjected to transposon mutagenesis using an *in vitro* Tn*5*-based transposition system (Epicentre, Madison, WI) with a chloramphenicol resistance (Cm^r; *cat*) cassette, and the insertion points were identified by sequencing individual insertions with primers within the cassette, as previously described (10–12). A clone with a nonpolar 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^r, 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 wild-type allele of *mpnC* into pRY107/28, which is pRY107 (13) containing the

Received 20 September 2012 Returned for modification 8 October 2012
Accepted 5 December 2012
Published ahead of print 17 December 2012
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doi:10.1128/IAI.01008-12

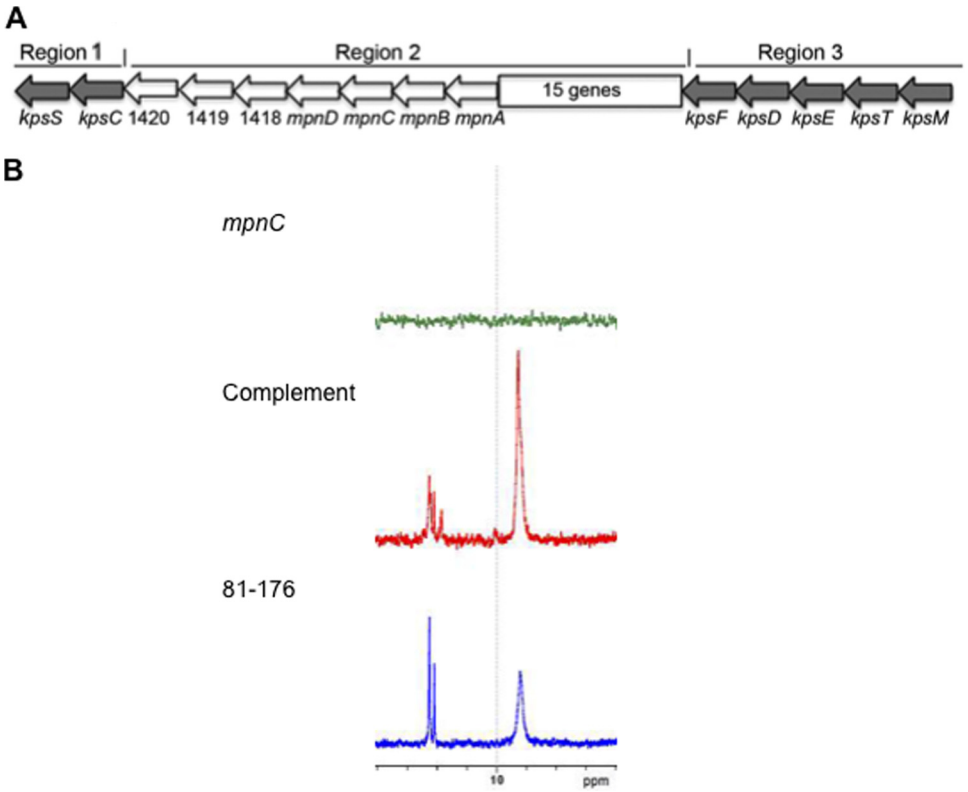

FIG 1 (A) Diagram of the capsule locus of strain 81-176. The locus is organized, like other class 2 capsule loci, into conserved regions 1 and 3, encoding proteins involved in capsule assembly and transport, and the variable region 2, encoding proteins involved in polysaccharide synthesis. The MeOPN biosynthesis genes (*mpnA* to *-D*) are found within region 2 but are highly conserved among strains if present and correspond to CJ1415 to CJ1418 in NCTC 11168 (8). 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 (16). 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 CPSs from wild-type 81-176 (blue), the *mpnC* mutant (green), and the *mpnC* mutant complemented in *trans* (red).

σ^{28} promoter of *flaA* cloned between the XbaI and BamHI sites. The wild-type *mpnC* gene was PCR amplified using high-fidelity polymerase (Clontech) and primers pg08.155 (5′-CGGGATCCGGTATAATGTGGCATATTGAAAGAG-3′) and pg08.150 (5′-CCGCTCGAGCTCTTAACTCATCTCCATCGAGATAAATAAG-3′), which 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 (14).

^{31}P NMR spectrometry. Preparations containing the CPSs were dissolved in D_2O, and ^{31}P nuclear magnetic resonance (NMR) was performed on a 400-MHz Bruker NMR instrument. Orthophosphoric acid was used as the external reference (δ 0.00).

Serum survival assays. Cultures (18 h old) of *C. jejuni* grown in MH biphasic media were washed and adjusted to an OD_{600} of 0.1 in minimal essential medium (MEM). Aliquots (100 μl) were added to wells of a 24-well plate containing 900 μl of prewarmed MEM 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 or 4 times.

Mouse infection experiments. BALB/c mice (7 to 8 weeks old; Jackson Laboratories, Bar Harbor, ME) were housed in groups of 10 with access to food and water *ad libitum*. For infection with *C. jejuni*, 1 liter of broth culture was harvested by centrifugation and resuspended in phosphate-buffered saline (PBS). The inocula were normalized by OD_{600} to ~10^{11} CFU/ml, and animals were inoculated intragastrically with 100 μl of the cell suspension. The inoculum doses were validated on MH agar plates prior to and immediately after infection of the animals. All animal experiments were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (15).

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 various time points following oral challenge, mice were sacrificed and small intestines were removed to recover lamina propria lymphocytes (LPLs) as described previously (16), 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 ~5-mm sections. Intraepithelial lymphocytes (IELs) were removed from these intestinal sections by placing the tissue in a solution of 1 mM dithiothreitol (DTT) and 1 mM EDTA at 37°C for two 20-min 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 medium. Following the digestion of small intestinal tissue sections, cells were pelleted by centrifugation and LPLs were isolated using a discontinuous (80 to 40%) Percoll gradient.

Intracellular cytokine staining. LPLs were cultured *in vitro* for 4 to 6 h in the presence of medium alone or phorbol-12-myristate-13-acetate (PMA) (Sigma; 20 ng/ml) and ionomycin (Sigma; 0.5 μg/ml). Medium was Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, nonessential 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 allophycocyanin (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 gamma interferon (IFN-γ) fluorescein isothiocyanate (FITC) (eBioscience) or anti-mouse interleukin 17 (IL-17) phycoerythrin (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. Human epithelial kidney (HEK) 293 cells are stably transfected with either human Toll-like receptor 4 (hTLR4), MD2, and CD14 (HEK-Blue-hTLR4) or hTLR2 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 FBS (Gibco) supplemented with 4.5 g/liter of glucose, 2 mM L-glutamine, 50 U/ml of penicillin, 50 μg/ml of streptomycin, 100 μg/ml of Normocin (InvivoGen), and 1× HEK-Blue selection (InvivoGen) in a 5% saturated CO_2 atmosphere at 37°C. The THP-1 cell line was maintained in standard RPMI 1640 medium with 10% heat-inactivated FBS (Gibco) supplemented with 4.5 g/liter of glucose, 2 mM L-glutamine, 1.5 g/liter of sodium bicarbonate, 10 mM HEPES, 1 mM sodium pyruvate, 50 U/ml of penicillin, 50 μg/ml of streptomycin, 100 μg/ml of Normocin (InvivoGen), 200 μg/ml of Zeocin (InvivoGen), and 250 μg/ml of G418 (InvivoGen) in a 5% saturated CO_2 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 the QUANTI-Blue colorimetric assay (InvivoGen). The assays were performed according to the manufacturer's protocols. Briefly, cells were seeded in a 96-well plate in triplicate (2.5×10^4 cells/well for HEK-Blue-hTLR4, 5×10^4 cells/well for HEK-Blue-hTLR2, and 1×10^5 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. Serial dilutions (10-fold) were prepared based on OD_{600} to yield the number of bacteria inoculated into each well. CFU were confirmed by plating serial dilutions on MH agar. Erythromyin (50 μg/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 of incubation, supernatants (20 μl) were transferred to a 96-well plate and incubated at 37°C with QUANTI-Blue (180 μl). SEAP activity was measured by reading the OD_{655} with a Synergy Mx multimode microplate reader (BioTek).

Statistical analyses. Differences in mouse colonization level, as assessed by the number of organisms shed ($\log_{10}$ CFU/gram of 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, and *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 proportions 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, NC), using a two-tailed alpha of 0.05.

Statistical analyses of complement killing, intracellular cytokine expression, and TLR assays were conducted using Student's *t* test. Differences were considered significant at a *P* value of <0.05.

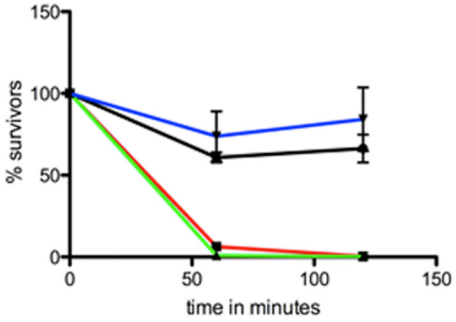

FIG 2 Sensitivities of 81-176 and mutants to complement killing by NHS. The percentages of survivors after 60 and 120 min of incubation with 10% NHS are shown. Black, 81-176; red, *kpsM* mutant; green, *mpnC* mutant; blue, *mpnC* mutant complemented in *trans*.

RESULTS

Construction of a mutant in the MeOPN biosynthetic pathway of 81-176. McNally et al. (8) identified the genes in *C. jejuni* strain NCTC 11168 (CJ1415 to CJ1418) 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 CPS of this strain. CJ1415 to 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 to CJ1418 in 81-176 are CJJ81176_1414 to CJJ81176_1417. Since the function of genes has been established in NCTC 11168, we have named the genes for MeOPN synthesis *mpnA* to *-D*, shown in Fig. 1A, for clarity in discussing these conserved genes in different strains. A mutant with a mutation in *mpnC* in 81-176 was shown to lack MeOPN by ^{31}P NMR, as predicted based on the NCTC 11168 data (8), and MeOPN was restored when the mutant was complemented (Fig. 1). The *mpnC* mutant produced CPS as determined by both NMR and immunoblotting with rabbit polyclonal antibody to whole cells of 81-176 (data not shown).

MeOPN contributes to serum resistance. There have been several reports demonstrating that nonencapsulated mutants of *C. jejuni* are more sensitive to normal human serum than wild-type strains (1, 17). Data comparable to those published for 81-176 and its isogenic *kpsM* mutant (1) 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, which lacked all CPS (Fig. 2). The *mpnC* mutant was significantly more sensitive than the wild type 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 those of the wild type (Fig. 2).

Capsule is required for prolonged mouse colonization. The abilities of the *kpsM* and *mpnC* mutants to colonize mice were compared to that of the wild type in a series of experiments. Animals were intragastrically infected with *C. jejuni*, and colonization was monitored postinfection by fecal shedding. Following infec-

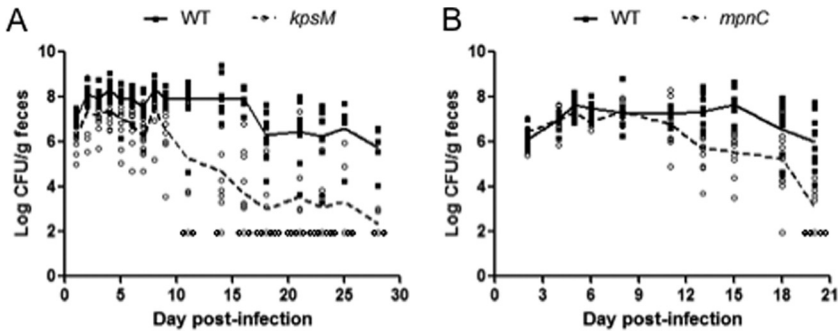

FIG 3 Colonization of BALB/c mice by *C. jejuni* strain 81-176 and various CPS mutants. The log CFU/g of feces shed by the wild type and either a *kpsM* mutant (A) or an *mpnC* mutant (B) are shown over the course of infection. Groups of 10 mice were intragastrically infected with ~10^{10} CFU. Each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

tion, wild-type *C. jejuni* 81-176 colonized mice on average at levels exceeding 10^6 CFU/g of feces (Fig. 3A). This high level of colonization was maintained for more 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 the wild type (Fig. 3A). In addition, the *kpsM*-infected mice had a shorter duration of colonization than did those infected with the wild type ($P = 0.06$). The majority of *kpsM*-infected mice cleared the infection by day 18, in contrast to the wild-type-infected mice, which remained colonized at some level through day 28 postinfection (the last day tested) (Fig. 3A). Thus, despite the similar levels of colonization seen early postinfection, by day 14, there was a statistically significant difference in colonization levels of mice infected with *kpsM* compared to those infected with the wild type ($P < 0.01$).

In parallel experiments, the colonization capacity of the *mpnC* mutant was compared to that of the wild type. Upon infection with the *mpnC* mutant, mice shed numbers of *C. jejuni* organisms in their stool similar to those shed by mice infected with the wild type (Fig. 3B). In fact, there were no significant differences in stool counts between the *mpnC* mutant and the wild type during the first 10 days postinfection, and only later in infection did the *mpnC* mutant demonstrate a significant reduction in the level of colonization compared to that of the wild type (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 those of the wild type and the *kpsM* mutant (Fig. 3).

IL-17 expression from intestinal T cells is modulated by the CPS. To determine if CPS had a role on immune responses *in vivo*, mice were orally infected with wild-type 81-176 and the *mpnC* or *kpsM* strain. At selected times postinfection, T cells were isolated from small intestine Peyer's patches, the epithelium, and the lamina propria. Following an *ex vivo* restimulation, the levels of expression of IL-17 and IFN-γ were determined using intracellular cytokine staining and flow cytometry. Figure 4A and B show representative histograms for CD4 LPLs and dot plots demonstrating gating strategies for intracellular cytokine staining. Following infection with either the *kpsM* or wild-type strain, CD4 cells from the lamina propria of *kpsM* mutant-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* mutant- or wild-type-infected mice at either time point. 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*. $CD4^+$ LPLs from *mpnC*-infected mice did not express significantly higher percentages of IL-17^+ cells on day 7 than did LPLs isolated from mice orally challenged with wild-type 81-176 (Fig. 4D). However, on day 21 postinfection, mice colonized by *mpnC* exhibited significantly higher percentages ($P < 0.01$) of $CD4^+$ LPLs expressing IL-17 than animals infected with the wild type. 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 signaling. 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 the wild type from 10^4 to 10^7 CFU for hTLR4 activation and from 10^5 to 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 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 the wild type and their complements (Fig. 6). For the *kpsM* mutant, these differences were observed from 10^4 to 10^7 CFU, and for the *mpnC* mutant, the differences were observed between 10^5 and 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 (18–23). Despite the lack of disease, the mouse model can provide information on traits required for colonization, the first step in pathogenesis (23). Here, we show that wild-type 81-176 colonizes BALB/c mice better than either an isogenic mutant lacking capsule (*kpsM* mutant) or a mutant expressing CPS without MeOPN (*mpnC* mutant). Interestingly, a reduction in colonization ability of an MeOPN-negative mutant of 81-176 (in the gene corre-

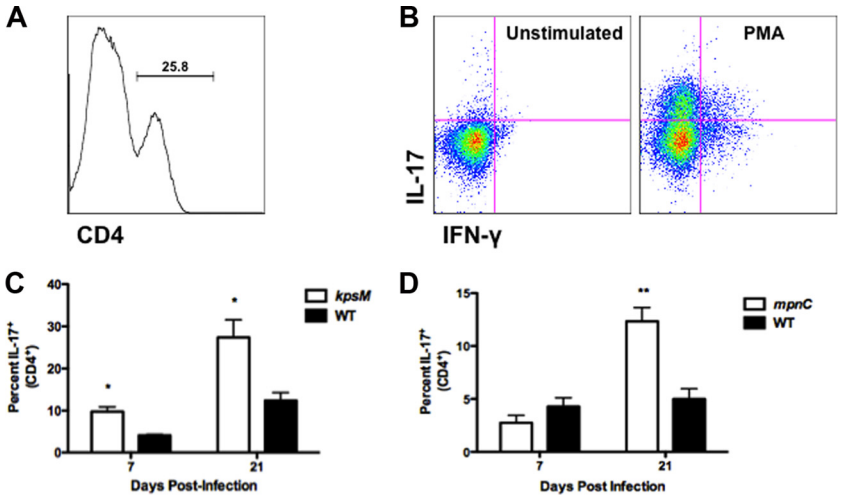

FIG 4 IL-17 expression is reduced in small intestinal $CD4^+$ LPLs from mice infected with wild-type *C. jejuni* 81-176. BALB/c mice (4 or 5/group) were orally infected with ~10^{10} CFU of *C. jejuni*. At days 7 and 21 postinfection, small intestines were removed and processed to isolate LPLs. LPLs were restimulated *in vitro* with PMA (20 ng/ml) and ionomycin (500 ng/ml) for 4 to 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 percentages 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* mutant or wild-type *C. jejuni*. (D) IL-17 expression in $CD4^+$ LPLs from mice infected with *mpnC* mutant or wild-type *C. jejuni*. Data represent the means ± standard errors of the means. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test). Data are representative of 2 or 3 independent experiments.

sponding to *mpnA*) compared to wild-type 81-176 was also reported with MyD88-defective mice (24). In our studies, BALB/c mice that were colonized with wild-type 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 those of the wild type, although statistical significance was reached 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 (25, 26).

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* mutant possessed higher levels of IL-17^+ $CD4^+$ cells in the small intestine than did mice colonized by the wild type 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 than was wild-type 81-176, the *kpsM* mutant appeared to show a greater reduction in colonization capacity than the *mpnC* mutant, suggesting that the presence of the 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 wild-type 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.

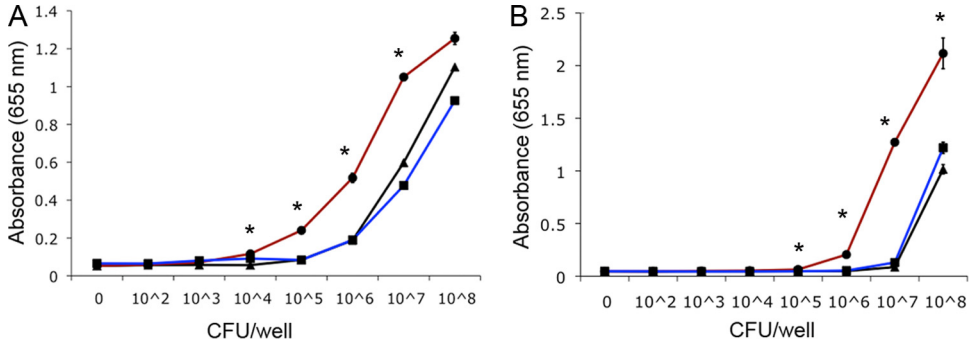

FIG 5 Activation of TLR4 in HEK 293 cells transfected with human TLR4-MD2-CD14 (A) and TLR2 in HEK 293 cells stably transfected with human TLR2-CD14 (B). TLR activation was monitored colorimetrically using a SEAP reporter gene placed under the control of an NF-κB inducible promoter. Tenfold serial dilutions 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 graphs are representative of three independent experiments. Asterisks indicate a *P* value of <0.005 compared to wild-type 81-176 or the complement. Black lines, wild-type 81-176; red lines, the isogenic *kpsM* mutant; blue lines, the *kpsM* mutant complemented in *trans*.

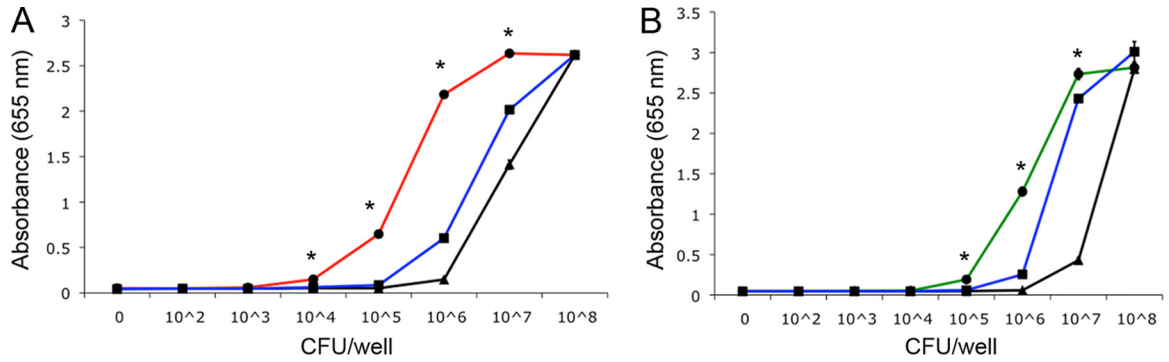

FIG 6 Activation of Toll-like receptors using a human monocytic (THP-1) reporter cell line by *kpsM* (A) and *mpnC* (B) mutants compared to wild-type 81-176 and complements of each mutant. Bacteria were added to cells as described in the legend to Fig. 5. Values represent the means and standard deviations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

T helper 17 (Th17) responses have come into focus due to their roles in maintaining intestinal homeostasis (27, 28) and protective immune responses against enteric pathogens (28–31). 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 (32, 33) and maintain a symbiotic balance between the microbiota and host (28). Specific animal vendors supply mice that are either colonized with SFB or not (Jackson Laboratories, SFB^-; Taconic, SFB^+) (33). These models can be exploited to evaluate innate or adaptive Th17 responses. Whereas iTh17 responses can be induced relatively quickly by cytokine signals (29, 30), adaptive Th17 responses occur later (days to weeks) (34) and are antigen-specific responses. In our present study, the Th17 responses likely represented an adaptive immune response, since Jackson Laboratory mice were used. Key to Th17 responses are the cytokines IL-17 and IL-22 and the upstream cytokines that lead to their expression, such as IL-1, IL-6, and IL-23 (34–36). IL-17 is primarily thought to be effective against extracellular pathogens by inducing inflammation and recruiting neutrophils to sites of infection (reviewed in reference 35). IL-22 exerts its protective effects by inducing epithelial cells to produce antibacterial molecules (37). Recently, Th17 responses have been demonstrated to have protective roles against *Salmonella* and *Citrobacter* infections in mice (29, 30). To date, only limited data exist regarding Th17 responses and *Campylobacter* infection. Edwards et al. (38) showed that cytokines involved in Th17 responses were induced in colon biopsy tissues following coculture 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 wild-type 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. (3), who showed that mutants of NCTC 11168 lacking CPS or MeOPN induced higher levels of IL-6, tumor necrosis factor alpha (TNF-α), and IL-10 from mouse dendritic cells than did the wild-type strain. Using dendritic cells from $TLR4^{-/-}$ mice, they also showed that some of these differences in cytokines were due to TLR4 signaling. Similar downregulation of the immune response has been observed for other bacterial capsules (39–44). 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 (45). The MeOPN modification on two distinct CPS structures of *C. jejuni* has now been shown to modulate cytokine responses and TLR signaling (3), 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 *Haemophilus influenzae* lipopolysaccharide with phosphorylcholine, which is also under phase-variable expression, enhances sensitivity to complement killing (46). The mechanism by which MeOPN interacts with components of the complement cascade is under investigation.

Collectively, these data indicate that 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 (47, 48). *C. jejuni* also expresses altered linkages of hydroxyacyl chains on lipid A that reduce TLR4 activation (49), and there is evidence that the *N*-linked glycan on proteins and certain lipooligosaccharide glycoforms can downregulate IL-6 induction (50). 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 (3). Here, we have demonstrated that a second *C. jejuni* CPS and the MeOPN modification modulate the immune response at multiple levels, including resistance to complement killing and cytokine induction via NF-κB signaling. 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 shed-

ding (51–53), 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 (54).

One of the hallmark characteristics of *C. jejuni* is its ability to undergo phase variation of surface antigens by slip strand mismatch repair (1, 7, 55–58). In terms of the CPS, this phase variation occurs at two levels. One is the high frequency on/off reversible expression that was originally described to occur in strain 81-176, such that a culture grown *in vitro* is a mixed population of encapsulated and unencapsulated variants (1). 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 nonstoichiometric 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 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.

ACKNOWLEDGMENTS

Work at NMRC was funded by NIAID R56 AI080593 (to P.G.) and Navy Work Unit 6000.RAD1.DA3.A0308. Work at UT Austin was funded by NIAID grants AI064184 and AI76322 and ARO grant 61789-MA-MUR (to M.S.T.). Work at the University of Guelph was funded by NSERC.

We thank Dawn Pattarini for technical assistance.

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or the U.S. government. P.G. and C.K.P. are employees of the U.S. government. This work was prepared as part of their official duties.

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