Effects of Sequential Campylobacter jejuni 81-176 Lipooligosaccharide Core Truncations on Biofilm Formation, Stress Survival, and Pathogenesis*†

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Campylobacter jejuni is a highly prevalent human pathogen for which pathogenic and stress survival strategies remain relatively poorly understood. We previously found that a C. jejuni strain 81-176 mutant defective for key virulence and stress survival attributes was also hyper-biofilm and hyperreactive to the UV fluorescent dye calcofluor white (CFW). We hypothesized that screening for CFW hyperreactive mutants would identify additional genes required for C. jejuni pathogenesis properties. Surprisingly, two such mutants harbored lesions in lipooligosaccharide (LOS) genes (waaF and lgtF), indicating a complete loss of the LOS outer core region. We utilized this as an opportunity to explore the role of each LOS core-specific moiety in the pathogenesis and stress survival of this strain and thus also constructed ΔgalT and ΔcstII mutants with more minor LOS truncations. Interestingly, we found that mutants lacking the LOS outer core (∆waaF and ∆lgtF but not ∆galT or ∆cstII mutants) exhibited enhanced biofilm formation. The presence of the complete outer core was also necessary for resistance to complement-mediated killing. In contrast, any LOS truncation, even that of the terminal sialic acid (∆cstII), resulted in diminished resistance to polymyxin B. The cathelicidin LL-37 was found to be active against C. jejuni, with the LOS mutants exhibiting modest but tiled alterations in LL-37 sensitivity. The ∆waaF mutant but not the other LOS mutant strains also exhibited a defect in intraepithelial cell survival, an aspect of C. jejuni pathogenesis that has only recently begun to be clarified. Finally, using a mouse competition model, we now provide the first direct evidence for the importance of the C. jejuni LOS in host colonization. Collectively, this study has uncovered novel roles for the C. jejuni LOS, highlights the dynamic nature of the C. jejuni cell envelope, and provides insight into the contribution of specific LOS core moieties to stress survival and pathogenesis.

The Gram-negative pathogen Campylobacter jejuni is the leading cause of bacterial food-borne diarrheal disease in the developed world, affecting up to (and sometimes more than) 1% of the population of North America, Europe, Australia, and New Zealand each year (4, 9, 79). Acute symptoms of C. jejuni infection include severe watery to bloody diarrhea, fever, nausea, and vomiting (12). Postinfectious sequelae can also occur, including the highly debilitating and sometimes fatal acute ascending bilateral paralysis Guillain-Barré syndrome (GBS), thought to occur in ~1 in 1,000 individuals infected with C. jejuni (35). Despite causing severe human disease, C. jejuni is a commensal in most other animal species (36). Up to 90% of commercial poultry products harbor live C. jejuni, and most cases of sporadic C. jejuni infection occur via consumption of undercooked poultry or cross-contamination of other food with raw poultry juice (36). C. jejuni is microaerophilic and capnophilic and requires rich media for growth and survival in the laboratory (81). Despite these fastidious attributes, C. jejuni can persist in unfavorable environments in nature, including water and milk, both of which are common sources of C. jejuni outbreaks (32, 48).

C. jejuni is polysaccharide rich, harboring four well-defined carbohydrate biosynthetic loci encoding proteins responsible for genesis of the lipooligosaccharide (LOS), capsular polysaccharide (CPS), O-linked flagellar sugars, and N-linked protein glycans (pgl) (21, 24, 46, 54, 65). The pgl system is well conserved among C. jejuni strains. In contrast, the LOS, CPS, and O-linked flagellar glycoproteins exhibit a high degree of interstrain variability, as evidenced by the extensive use of LOS and CPS antigens as strain serotyping systems. Many genes in these hypervariable regions are also subject to phase variation, further confounding immune system responses to C. jejuni infection.

The C. jejuni LOS comprises two main components: the hydrophobic lipid A anchor and an oligosaccharide consisting of a conserved inner core and a variable outer core (22). C. jejuni LOS lacks the O-antigen characteristic of lipopolysaccharides (LPS) found in other bacterial species. LPS and LOS participate in the pathogenesis of numerous Gram-negative bacteria, acting as endotoxins, adherence factors, factors that maintain the stability of the outer membrane and protect cells from environmental stresses, and host defense factors (19, 73, 86). Currently, the best-characterized contribution of the C. jejuni LOS to infection is its role in resistance to complement-mediated killing (34). The acetylated lipopolysaccharide (LPS) found in other Gram-negative bacteria participates in the pathogenesis of Gram-negative bacteria, acting as endotoxins, adherence factors, factors that maintain the stability of the outer membrane and protect cells from environmental stresses, and host defense factors (19, 73, 86).
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**C. JEJUNI LOS CORE RESIDUES IN PATHOGENESIS**

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*jejuni* LOS to human disease is its relationship to the debilitating neuropathy GBS, in which antibodies mounted against certain ganglioside-mimicking LOS structures (21, 60, 82) cross-react with and attack gangliosides on peripheral nerves (3, 6, 85). LOS structures for many *C. jejuni* strains have now been elucidated, as have genes required for LOS biosynthesis (21). The LOS structure of our laboratory strain, 81-176, is shown in Fig. 1A; enzymes involved in its biosynthesis will be described in more detail in Results. Depending on phase variation of the *cgtA* gene, the 81-176 LOS can mimic GM2, GM3, GD1b, and GD2 gangliosides (21). Various *C. jejuni* LOS mutants have also been found to exhibit enhanced sensitivities to certain antimicrobial substances (37, 43, 53, 56), and two severely truncated mutants (a strain 81-176 Δ*waaC* mutant and a strain 11168 mutant with a large LOS gene cluster deletion) are defective for host cell invasion (43, 56). However, the latter two mutants also produce altered CPS and lipid A, respectively (42, 56), and an invasion defect previously ascribed to a Δ*waaF* mutant (42) was subsequently shown to be due to sensitivity of that mutant to the detergent used for host cell lysis (43). Thus, although previous work has shown that the *C. jejuni* LOS is important as a pathogenesis factor, the relative contributions of specific oligosaccharide core structures to particular pathogenesis-related properties have not been described, nor has a direct role for the LOS in host colonization been demonstrated.

Biofilms are surface-associated, dynamic consortia of microorganisms encased in a protective polymeric matrix (26). It is now thought that in nature, >99% of bacteria exist in biofilms rather than as free-swimming (planktonic) cells. Biofilm residents exhibit important survival differences from planktonic cells, including altered metabolism, physiology changes, and increased stress tolerance (67). The biofilm lifestyle is a key contributing factor to *C. jejuni*’s prevalence and ability to withstand stressful environments in nature despite fastidious growth requirements (81). Our understanding of *C. jejuni* biofilms lags behind that of other bacteria; however, recent work has identified several genes involved in regulation and other aspects of biofilm dynamics (14, 18, 39, 40, 57, 75, 80). One of these genes controls a global stress response known as the stringent response (SR) (20); unexpectedly, a Δ*spoT* mutant incapable of mounting an SR also exhibited a dramatic hyper-biofilm phenotype (57). Biofilm upregulation in the Δ*spoT* mutant occurred concomitantly with increased reactivity to calcifluor white (CFW), a UV fluorescent dye that reacts with β-1,3- and β-1,4-linked polysaccharides on cell surfaces, suggesting that such a polysaccharide may be a component of the biofilm matrix.

Despite its prevalence, much less is understood about the pathogenesis of *C. jejuni* than that of prototypical enteric bacteria such as *Escherichia coli* and *Salmonella* spp. Furthermore, although several well-annotated *C. jejuni* genome sequences have been published, in silico efforts to identify pathogenesis determinants have been frustrating, with none of the sequenced *C. jejuni* genomes revealing obvious pathogenicity islands, type III “injection-like” secretion systems, or other hallmark virulence determinants (31, 72). As such, screens and selections are critical for identifying *C. jejuni* genes important for modulating pathogenesis-associated properties.

As noted, we previously found that the Δ*spoT* mutant exhibited a dramatic hyper-biofilm phenotype (57). Although the precise nature of the concomitantly upregulated CFW-reactive polymer remains unknown, initial studies suggested that it was not composed of previously described polysaccharides (57). We also previously demonstrated that the SR is important for specific *C. jejuni* virulence- and stress-related attributes (20).
Collectively, this led us to rationalize that screening a random transposon (Tn) library for C. jejuni mutants exhibiting a CFW-hyperreactive phenotype would uncover genes important for biofilm dynamics and other key aspects of C. jejuni pathogenesis and survival. Somewhat unexpectedly, two such mutants harbored insertions suggesting complete loss of the LOS outer core. We took advantage of this finding to generate mutants defective for each of the other LOS core-specific enzymes in 81-176, with the goal of assigning specific roles in pathogenesis properties to distinct moieties of the LOS. Consequently, this study has identified novel roles for LOS sugars in modulating biofilm dynamics, complement sensitivity, resistance to two classes of antimicrobial peptides, and intracellular survival. We also present the first direct evidence of a role for the C. jejuni LOS in host colonization.

### Materials and Methods

**Bacterial strains and growth conditions.** *C. jejuni* strain 81-176, originally isolated from an outbreak of campylobacteriosis after consumption of raw milk (48), was the wild-type strain for these studies. All *C. jejuni* strains were grown at 38°C on Mueller-Hinton (MH) agar or broth (Oxoid) supplemented with vancomycin (10 μg/ml) and trimethoprim (5 μg/liter) (MH-TV) under microaerobic and capnogenic conditions (6% O2 and 12% CO2). *E. coli* DH5α was used for plasmid construction and was grown on Luria-Bertani broth (LB; Sigma) at 37°C. When necessary, media were supplemented with kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), or ampicillin (100 μg/ml). *C. jejuni* mutant strains used in this study are listed in Table 1.

**Random in vitro Tn mutagenesis of C. jejuni using the mariner transposon.** (i) Purification of MBP-Himari. The MBP-Himari Tn transposase was purified according to a modified protocol from Akeryle and Lampke (1) and instructions from New England Biolabs (NEB) (63a).

An overnight culture of *E. coli* TB1 containing the plasmid pMALC9 (1) grown in LB and ampicillin (100 μg/ml) at 37°C was subcultured 1:50 into 100 ml fresh LB containing ampicillin and 0.2% (wt/vol) glucose, and growth at 37°C was continued. At an approximate optical density at 600 nm (OD600) of 0.5, protein concentration was determined by the Bradford assay (Bio-Rad) (approximately 0.16 mg/ml), and purity was assessed by sodium dodeyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

(ii) In vitro transposon mutagenesis. In vitro transposon reactions were performed as described by Harrison et al. (28) and Akeryle and Lampke (1), except that DNA was purified using Qiagen DNeasy columns following transposition and prior to ligation. The DNA from the ligation reaction was dialyzed on a 0.025 μm hydrophobic filter floating on distilled water (dH2O) for 20 min and then transformed by natural transformation into *C. jejuni* 81-176. Kanamycin-resistant (solo Tn from pFalcon) and chloramphenicol-resistant (picard Tn from pEnterprise) clones were selected on MH plates supplemented with the appropriate antibiotics. Approximately 3,000 to 4,000 single colonies from each round of mutagenesis were directly harvested from the plates. A total of 4 rounds of mutagenesis were carried out to construct the library. To confirm random Tn insertion, 10 of the colonies from each of the solo and picard Tn libraries were screened by Southern analyses (data not shown).

**CFW screening.** Wild-type *C. jejuni* 81-176, 81-76:sofo Tn library, and 81-176:picard Tn library strains were taken directly from the freezer and grown for 2 days on MH-TV plates. Strains were diluted to an OD600 of 0.1. One hundred microliters of 10^-3 dilutions were plated on brain heart infusion (BHI; BD) agar plates supplemented with antibiotics and 0.002% CFW (fluorescent brightener 28; Sigma) and incubated for 2 days in the dark. Plates were inspected under long-wave UV light, and hyper-fluorescent colonies were patched onto MH plates and grown for 1 day. Each clone was then again patched onto BHI-CFW plates and incubated for 2 days in the dark, and hyper-fluorescence was observed under long-wave UV light. To confirm the presence of the CFW hyperreactive phenotype with the transposon insertion, genomic DNA was prepared from the mutant strains using the Wizard genomic DNA purification kit (Promega) and reintroduced into wild-type 81-176 by natural transformation and selection on the appropriate antibiotic. The transformed strains were grown in shaking MH broth culture to early log phase and diluted to an OD600 of 0.2, and 10 μl was spotted onto BHI-CFW plates. To avoid any impact of the screening procedure on phenotypic analyses of the Δgft retransformed strain, linkage analysis was used for all subsequent assays and complementation. The spotting technique described above was also used to generate the CFW plate data described in Results.

**Transposon mapping via random PCR.** Using the genomic DNA described above, the region flanking the transposon insertion sites were amplified using the CEKG technique discussed by Salama et al. (76). First, the primers CEKG2A, CEKG2B, and CEKG2C were used with the mariner-2 or mariner-3 primer to amplify transposon-flanking sites (see Table S1 for all primer sequences except the CEKG primers, which are found in reference 76). A second PCR was performed on the product of the first PCR using the primers CEKG4 and mariner-IR-1. The amplicons were purified and sequenced using the primer MarOut3. Fine mapping to determine the precise location of the transposon for the Δgft Tn library strain and Δgft Tn library strain corresponding to the insert region amplified using the primers brt1-Tn-F and brt1-Tn-R for the Δgft-Tn muta- tion and brt28-Tn-F and brt28-Tn-R for the Δgft-F mutant. The Δgft-F mutant was constructed by PCR amplification of waaf from 81-176 genomic DNA using the primers pGEM-waaf-F and pGEM-waaf-R. The resulting amplicon was cloned and sequenced using the primers pUC18K-2 (58), carrying a nonpolar kanamycin resistance (apha-3) cassette, which was each digested with XbaI and KpnI enzymes and ligated to form the plasmid pGEM-waaf-F and pGEM-waaf-R. The resulting amplicon and the plasmid pUC18K-2 (58), carrying a nonpolar kanamycin resistance (apha-3) cassette, were each digested with XbaI and KpnI enzymes and ligated to form the plasmid pGEM-waaf-F and pGEM-waaf-R. This plasmid was delivered into 81-176 via natural transformation.

### Results

**Constitution of ΔwaaF, ΔgftT, and Δsclf1 mutants and ΔwaaF-c, Δgftc-c, and Δgft-Tc complemented strains.** The ΔwaaF mutant was constructed by PCR amplification of waaf from 81-176 genomic DNA using the primers waaf-F and waaf-R and by cloning the PCR product into the commercial vector pGEM-T (Promega). Inverse PCR was performed on the resulting vector using the primers pGEM-waaf-F and pGEM-waaf-R. The resulting amplicon and the plasmid pUC18K-2 (58), carrying a nonpolar kanamycin resistance (apha-3) cassette, were each digested with XbaI and KpnI enzymes and ligated to form the plasmid pGEM-waaf-F and pGEM-waaf-R. This plasmid was delivered into 81-176 via natural transformation, and kanamycin-resistant colonies were isolated. ΔwaaF mutants were confirmed via PCR analysis and sequencing. The Δgft c mutant was constructed in the same manner as the ΔgftF mutant except that the initial primers were gat-F and gat-R, and inverse PCR was performed using the primers pGEM-gat-F and pGEM-gat-R.

The Δsclf1 mutant was constructed as a control for a previous study. Briefly, the ctt1 gene from *C. jejuni* strain ATCC 43446 was amplified in two stages using the primers CJ-131, CJ-269, CJ-132, and CJ-268. CJ-131 and -269 were the primers used to amplify the 5′ region of ctt1, and CJ-132 and -268 were the primers used to amplify the 3′ region of ctt1. This was done to insert KpnI and SaeI sites in the middle of the gene. A final PCR using CJ-131 and CJ-132 was performed to amplify the full-length ctt1 gene, containing two restriction sites in the middle, an NdeI site at the 5′ end, and a SalI site at the 3′ end. The amplicon was then inserted into the plasmid pCWori+ (lac-Z), giving the plasmid pCST-60.

### Table 1. C. jejuni strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Descriptiona</th>
<th>Reference</th>
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<tbody>
<tr>
<td>81-176</td>
<td>Wild type</td>
<td>48</td>
</tr>
<tr>
<td>ΔwaaF+</td>
<td>81-176 waaf^-waaf::Tn solo</td>
<td>This study</td>
</tr>
<tr>
<td>ΔwaaF-</td>
<td>81-176 waaf::apha-3</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgftT+</td>
<td>81-176 gft::apha-3</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgftT-</td>
<td>81-176 gft::apha-3 rm-cat::waafF</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgftF-c</td>
<td>81-176 gft::apha-3 rm-apha-3-3::gftF</td>
<td>This study</td>
</tr>
<tr>
<td>ΔgftT-c</td>
<td>81-176 gft::apha-3 rm-cat::gatT</td>
<td>This study</td>
</tr>
</tbody>
</table>

a: Tn solo, apha-3, and rm-apha-3 confer kanamycin resistance. Tn picard and rm-cat confer chloramphenicol resistance.

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*Downloaded from [http://jb.asm.org/](http://jb.asm.org/) on March 26, 2021 by guest*
The apfA-3 gene, encoding a kanamycin resistance cassette, was introduced into pCST-00 using the KpnI and SacI sites. The resulting plasmid, designated pCST-72, was electroporated into C. jejuni strain 81-176, and ΔlgtF colonies were isolated. Positive clones were verified by PCR and sequencing.

For complemented strains, the waaF gene was PCR amplified from 81-176 genomic DNA using the primers pR-waaF-F and pR-waaF-R. The waaF ampli-
con was digested with MfeI and XbaI and ligated into pRRC (45), which had been digested with the same enzymes, to produce pRRC-waaF. The galT gene was PCR amplified from 81-176 genomic DNA using the primers pR-galf-F and pR-galf-R, and the lqtG gene was amplified using the primers pR-lqtG-F and pR-lqtG-R. The resulting galT and lqtG amplicons were digested with XbaI and ligated into XbaI-digested pRRC and pRRK, respectively, to produce the pRRC-galT and pRRK-lqtG plasmids. The correct orientation of the genes in the plasmids was confirmed, after which the plasmids were naturally transformed into the respective mutants, the ΔlgtF, ΔlqtG, and ΔlgtF mutants. Recombina-
tion was confirmed via PCR. The pRRC and pRRK plasmids were kindly pro-
vided by Brendan Wren (45) and Julian Ketley.

C. jejuni LOS analysis by SDS-PAGE. LOS samples were prepared from whole-cell lysates using a modified method described by Hitchcock and Brown (29). Briefly, cells, standardized to an OD600 of 5.0, were resuspended in lysis buffer (2% SDS, 4% β-mercaptoethanol, 10% glycerol, 1.0 M Tris [pH 6.8], bromophenol blue) and heated for 5 min at 95°C. Samples were then treated with proteinase K and incubated overnight at 55°C. For silver stain analyses, LOS preparations were heated for 5 min at 95°C and 10 µl of each sample was separated via 15% SDS-PAGE. The resulting gels were stained with silver as described previously (83) and developed with Bio-Rad silver stain developer (Bio-Rad).

Isolation of LOS and MS analysis. A half plate of confluent bacteria was resuspended in 0.3 ml of phosphate-buffered saline (PBS). One millilitre of 100% ethanol was then added, mixed, and allowed to stand for 1 h at room temperature. Cells were harvested, and the pellet was washed twice in 100% ethanol, twice in 100% acetone, and allowed to air dry. The intact LOS of the

strains was measured at 550 nm using a spectrophotometer (Thermo Electron Co.).

Sensitivity assay. Sensitivity to complement-mediated killing was as-
sayed by a slightly modified method of Querry et al. (23). Mid-log-phase over-
night broth cultures were diluted in PBS to a concentration of 10 CFU/ml and incubated in pooled human serum (10% serum as the final concentration) for 40 or 80 min at 38°C under standard C. jejuni growth conditions. At the specified time points, bacterial survival was assessed via CFU enumeration on MH plates. Strains were also incubated with pooled human serum that was heat inactivated at 60°C for 1 h as a control.

Sensitivity to antimicrobial peptides, SDS, and EDTA. The MICs of LL-37, polymyxin B (Sigma), SDS (Fisher Scientific), and EDTA (Sigma) for the strains were determined using a microtiter broth dilution method (52) in MH broth and an initial inoculum of 10⁵ cells/ml (diluted from overnight mid-log-phase cul-
tures). Polypeptide microtiter plates containing bacterial strains with the vari-
ous substances were incubated for 48 h at 38°C under standard C. jejuni growth conditions, and dilutions were spotted on MH plates for survivability. LL-37 was kindly provided by R. E. W. Hancock. Shown are the results of a representative experiment of at least three independent repeats on different days.

Adherence, invasion, and intracellular survival assay in Caco-2 cells. Bacterial infections in Caco-2 intestinal epithelial cells were performed as previously described (20), except that shaking MH broth mid-log-phase bacterial cultures were used instead of biphasic cultures.

Mouse colonization. BALB/cByJ mice from Jackson Laboratories (Bar Har-
bor, ME) were maintained in ABSL-2 housing in the Division of Lab Animal Services at the Medical College of Georgia, with five mice per experimental group. Each mouse was infected with a mixture of 5 × 10⁷ CFU of the wild type and either the C. jejuni ΔlgtF mutant or ΔlgtFΔwaaF mutant via oral gavage as previously described (69). C. jejuni organisms shed in fecal pellets from each mouse at 7, 14, and 21 days postinfection were homogenized and enumerated on
with \textit{C. jejuni} biofilm formation. For reference, the CFW profile of each mutant is shown in Fig. 3A, with the 81-176 wild type and the \(\Delta poT\) CFW hyperreactive strains shown as controls. Although the \(\Delta galT\) mutant was very modestly CFW hyperreactive and the \(\Delta cstII\) mutant was modestly hyporeactive, only the \(\Delta waaF\) and \(\Delta lgtF\) mutants consistently exhibited significant differences from the wild type, with each mutant displaying clear CFW hyperreactivity (Fig. 3A).

Biofilm formation was assessed using a previously established standing culture assay followed by crystal violet staining and spectrophotometric quantification of triplicate biofilms (54, 68). Shown are results from a representative experiment from multiple experimental trials (Fig. 3B). Consistent with CFW reactivity profiles, the \(\Delta lgtF\) and \(\Delta waaF\) mutants also exhibited a statistically significant increase in biofilm formation compared to that of the wild type at 2 and 3 days postinoculation. In contrast, the \(\Delta galT\) and \(\Delta cstII\) mutants did not exhibit a difference in biofilm formation from the wild type, nor did the \(\Delta lgtF\)-c and \(\Delta waaF\)-c complemented strains. To investigate whether growth differences might account for the biofilm observations and other attributes described below, we also assessed the growth of each mutant strain in standard shaking broth cultures. None of the mutants exhibited growth or survival defects compared to the wild type by either OD\(_{600}\) or CFU/ml analyses during normal growth curves in vitro (Fig. 3C and D).

**The LOS outer core is important for protecting \textit{C. jejuni} from complement-mediated killing.** To begin to assess the role of specific LOS moieties on host-related properties, we investigated the ability of our mutant and complemented strains to survive 40- and 80-min exposures to 10% pooled human serum. Only the \(\Delta lgtF\) and \(\Delta waaF\) mutants exhibited statistically significant defects in serum resistance compared to the wild type (Fig. 4), with \(\Delta waaF\) mutant recovery near the detection limit after 40 min of incubation. Serum preincubated at 60°C (heat killed) abrogated all killing (Fig. 4, bracketed group 1). The \(\Delta galT\) and \(\Delta cstII\) mutants and all complemented strains exhibited sensitivity levels similar to that of the wild type (Fig. 4, bracketed group 2).

**LOS truncations result in modest, tiled sensitivities to LL-37 and hypersensitivity to polymyxin B.** Because the LOS is a major component of the \textit{C. jejuni} cell envelope and a target of antimicrobial peptides (AMPs), we next explored the sensitivity of the LOS mutants to AMPs. Determination of the MICs of LL-37 and polymyxin B (PxB) for wild-type \textit{C. jejuni} 81-176 and the LOS mutants revealed several unexpected findings. First, while the MIC of LL-37 for our wild-type strain was 5.68 \mu g/ml, the serially truncated LOS mutants yielded titled LL-37 MICs, with the \(\Delta waaF\) (1.42 \mu g/ml) and \(\Delta lgtF\) (2.40 \mu g/ml) mutants exhibiting modestly lower MICs than the wild type and the \(\Delta galT\) (8.08 \mu g/ml) and \(\Delta cstII\) (12.13 \mu g/ml) mutants unexpectedly exhibiting modestly higher MICs than the wild type (Table 2). In contrast, all of the mutants displayed a significant (>15-fold) decrease in MIC for PxB compared to that of the wild type (3.13 \mu g/ml), with the various LOS mutants displaying modest differences from each other in a trend opposite to that observed for LL-37 (ranging from 0.21 \mu g/ml for the \(\Delta waaF\) mutant to 0.06 \mu g/ml for the \(\Delta cstII\) mutant) (Table 2).

As the AMP data suggested general alterations in the cell wall, we also explored the sensitivity of the mutants to deter-
gents, salts, and chelators and investigated cell surface hydrophobicity and general profiles of outer membrane proteins. No differences in sensitivity to Tween 20, deoxycholate, or sodium chloride were observed (data not shown); however, each LOS mutant exhibited a 2-fold decrease in its MIC of SDS (Table 2). No overt differences were observed for EDTA sensitivity (Table 2), outer membrane protein profiles (data not shown), or surface hydrophobicity utilizing hexadecane- and ammonium sulfate-based assays (11, 37; data not shown).

The ΔwaaF mutant exhibits a defect in intracellular survival. Previous work indicated that mutants defective specifically for the LOS (i.e., the ΔwaaF, ΔlgTF, ΔgalT, and ΔcstII mutants) were not defective for invasion of host cells in vitro (25, 43). As intracellular survival provides another recently

![FIG. 3. CFW reactivity, biofilm formation, and broth growth properties of LOS mutant strains.](image)

![FIG. 4. Complement-mediated killing of LOS mutant strains.](image)
recognized measure of *C. jejuni* pathogenic potential, we also assessed the ability of each of our mutant strains to survive inside Caco-2 intestinal epithelial cells for 24 h postinoculation. Consistent with previous observations, none of our LOS mutants exhibited an invasion defect (Fig. 5 and data not shown). Furthermore, the ΔcstII, ΔgalT, and ΔlgtF mutants did not display differences from the wild type in intracellular survival (data not shown). However, the ΔwaaF mutant exhibited a reproducible and statistically significant defect (P < 0.05) for intracellular survival (Fig. 5A), with only 11% of the bacteria recovered relative to wild-type levels 24 h after inoculation (Fig. 5B). Complementation restored the intracellular survival defect to near-wild-type levels (Fig. 5A and B).

A mouse competition model identifies a role for the *C. jejuni* LOS in colonization in vivo. To date, little is known regarding participation of the *C. jejuni* LOS in host colonization. To explore this, we tested our two most truncated mutants, the ΔlgtF and ΔwaaF mutants, in a recently described mouse competition model for *C. jejuni* colonization (69). BALB/c ByJ mice were infected orogastrically with a mixture of bacteria containing equal doses of the wild-type and mutant *C. jejuni* strains. Fecal pellets were harvested at 7, 14, and 21 days postinfection and plated for bacterial counts on selective and nonselective plates. Both the ΔlgtF mutant (Fig. 6A) and the ΔwaaF mutant (Fig. 6B) exhibited a striking and statistically significant colonization defect evident from 7 days postinfection. Both the ΔwaaF and ΔlgtF mutants grew comparably on selective and nonselective plates used to assess levels of mutant versus that of the wild type following colonization, and each mutant exhibited wild-type motility (data not shown). Furthermore, when mutant and wild-type strains were grown together in shaking broth culture, neither the ΔlgtF nor the ΔwaaF mutant exhibited a growth defect in competition with the wild type (Fig. 6C and D), indicating that the colonization defect likely reflects in vivo-specific phenomena.

**DISCUSSION**

This study, initiated following an unbiased screen to identify *C. jejuni* genes likely to be important for biofilm formation, stress survival, and virulence-associated attributes, has resulted in the delineation of novel roles for the *C. jejuni* LOS in a number of key pathogenesis properties. Our in-depth comparison of serially truncated LOS core mutations in a single highly invasive and virulent strain, 81-176, has also established for the first time clear cutoff points for the LOS in modulating certain pathogenic traits (i.e., biofilms, complement resistance, and intracellular survival) and both all-or-nothing and graded effects of specific LOS moieties on other attributes (i.e., resistance to different AMPs). We also present the first direct evidence of a role for the *C. jejuni* LOS in colonization.

As noted, bacterial biofilms play significant roles in infectious disease and confer advantages over bacteria in the planktonic state, including enhanced stress survival and antibiotic resistance. For a fastidious yet prevalent organism like *C. jejuni*, a biofilm lifestyle is especially important for surviving unfavorable conditions (81). Among our LOS mutants, only the ΔlgtF and ΔwaaF mutants exhibited a hyper-biofilm phenotype, while the ΔgalT and ΔcstII mutants were similar to the wild type, suggesting a role for the LOS outer core in maintenance of planktonic growth and/or biofilm dispersal. Although this is a new finding for *C. jejuni*, the LPSs and LOSs of other bacteria have also been shown to interface with biofilm production. Some groups reported findings similar to ours, whereby LPS/LOS mutations result in enhanced biofilms and/or exopolysaccharides involved in biofilm formation (59, 71, 78), while others reported that LPS defects result in diminished biofilms (10, 63). Still others observed mixed biofilm results for LPS mutants depending on the incubation conditions (7). Together with our prior observation that a *C. jejuni* kpsM (CPS export) mutant is also CFW hyperreactive and hyper-biofilm forming.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC of:</th>
<th>LL-37 (µg/ml)</th>
<th>Polymyxin B (µg/ml)</th>
<th>SDS (µg/ml)</th>
<th>EDTA (mM)</th>
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<tr>
<td>81-176</td>
<td></td>
<td>5.68</td>
<td>3.13</td>
<td>248</td>
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<td></td>
<td>1.42</td>
<td>0.21</td>
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* Complemented strains had MICs equivalent to that of wild-type 81-176.
LOS results are consistent with studies of Neisseria spp., with which it has been shown that MBL binds bacteria more strongly when the LOS is truncated (34) and that loss of LOS phosphoethanolamine (PEtn) results in enhanced complement killing (51). Similar mechanisms may occur in C. jejuni and await further study.

Although the entire outer core was required for optimal protection from complement, the ΔwaaF mutant was more serum sensitive than the ΔlgtF mutant, suggesting an important role for the inner core HepII moiety in this attribute. Interestingly, we have also now found that the ΔwaaF deep rough mutant is unique among our LOS core-specific mutants in exhibiting a defect in long-term survival of C. jejuni inside epithelial cells, an increasingly recognized marker for C. jejuni pathogenesis (14, 20, 86). Several previous studies had suggested a role for the LOS in the C. jejuni-host cell interaction, but concrete evidence for a role of the core oligosaccharide had not been elucidated. For instance, one study assessing the invasion and colonization potential of different C. jejuni strains suggested the enhanced presence of LOS-relevant genes cgtB and wlaN in invasive strains (61); however, mutants harboring gene deletions were not examined. Other studies indicated that three LOS mutants exhibited diminished invasion capacities, but these likely reflect pleiotropic and non-LOS core-specific effects. For example, one study involved a mutant deleted for a large LOS locus containing htrB (56). HtrB is involved in lipid A biosynthesis, and the large deletion mutant exhibited a growth defect (56) suggesting significant phenotypic and genotypic differences from our targeted core mutants. Another study reported that a ΔwaaC mutant is de-
fective for invasion (41); however, waaC also participates in biosynthesis of the CPS, which itself influences invasion of C. jejuni into epithelial cells in vitro (8, 42). A third published invasion defect was subsequently shown to be due to the detergent sensitivity of the ΔwaaF mutant studied (41, 43). We routinely utilize a water-based lysis procedure for harvesting bacteria from cell infections (20) which does not affect survival of the ΔwaaF mutant (43; our unpublished observations). Our ΔwaaF mutant intracellular survival findings are also not likely due to defects in CPS production, as (i) Western blots with Penner and CPS antisera yielded wild-type profiles (unpublished observations), (ii) WaaF was previously shown to be independent of the CPS biosynthesis pathway (66), and (iii) CPS alterations are expected to yield invasion defects (8) absent from our LOS mutants. Together, these findings indicate that WaaF joins other C. jejuni factors recently identified as important for impacting intraepithelial cell survival, which now also include the SR, the FeoB iron uptake protein, the enzyme PPK1, the CprS sensor kinase, and anaerobic adaptation (14, 20, 62, 80, 84).

AMPs are a critical component of the host innate immune defense against invading pathogens. PxB binds negatively charged structures such as the LPS/LOS, displaces calcium and magnesium ions, disrupts the outer membrane, and promotes self-uptake (87). LL-37 is a human cathelicidin active against Gram-negative and Gram-positive bacteria, binding the cell surface via electrostatic interactions to promote membrane leakage (23, 64, 77). Each of our LOS mutants was significantly defective for PxB resistance. This included the very modestly truncated acsiII mutant, implicating a role for the sialic acid component not only in GBS but also in protection from PxB. In contrast, the LOS mutants exhibited only moderate (4-fold or lower) differences in LL-37 MICs compared to the wild type, with an interesting tiled pattern observed for the truncation series. These observations suggest cell envelope perturbations despite the fact that we were unable to detect overt changes in outer membrane protein profiles or cell surface hydrophobicity. One potential explanation for the latter is that C. jejuni 81-176 harbors an extensive CPS, including an additional α-glucan capsule (70), that may interfere with detection of hydrophobicity alterations associated with LOS truncations. Nonetheless, consistent with envelope perturbations, our LOS mutants exhibited SDS sensitivities similar to those reported for the ΔwaaF mutant and the large LOS deletion mutants of strain 11168 (37, 56), and our AMP observations are consistent with a recent E. coli study hypothesizing that negative cell surface charges normally buried by the LPS/LOS are exposed in LPS/LOS mutants and thereby more available to interact with cationic molecules (5). Previous studies of very severe LOS truncations in C. jejuni also implicated roles for the LOS in PxB resistance (37, 53, 56); however, our work provides the first evidence of the importance of LgtF, GalT, and CstII in this aspect of innate immunity and the first demonstration of an antimicrobial effect of LL-37 toward C. jejuni. These and other findings described above also again reflect our increasing appreciation for cell envelope dynamics and the likely involvement of feedback loops and as-yet-unidentified regulatory mechanisms in these processes. For instance, C. jejuni AMP resistance also involves efflux pumps (2, 27), surface expression or function of which may be altered due to secondary or tertiary effects of the LOS mutations. The CFW observations presented here and previously (57) also illustrate that alterations to envelope polysaccharides can cause compensatory changes in other envelope components, which in turn may affect attributes like AMP resistance, electrostatic interactions, and surface hydrophobicity.

Finally, this study provides the first evidence of a role for the C. jejuni LOS in host colonization. Previous work suggested a correlation between the presence of an LOS gene (cgtB, encoding a β-1,3-galactosyltransferase) and the colonization potential of clinical C. jejuni isolates (61); however, as noted above, targeted gene deletions were not tested, and the structure of the 81-176 LOS does not suggest the activity of CgtB in LOS biosynthesis (21). Our observations now clearly establish that, as with other C. jejuni polysaccharides like the CPS (38), plg (44), and flagellar glycosylation (33) systems, the LOS is an important component in host colonization. The mouse data also highlight the utility of mouse competition models to assess the ability of C. jejuni both to colonize the intestinal tract (31, 69) and to disseminate systemically into deeper tissues (30). The utility of these models is additionally important given a recent study showing unpredictable variability in competition studies in the more traditional chicken model of colonization (16). Future work to explore additional LOS mutants of 81-176 and other C. jejuni strains should lend even more insight into the role of this structure in colonization. The animal and biofilm data also touch on the question of whether C. jejuni biofilms are important in vivo. While evidence presented here might suggest not, it should also be noted that every C. jejuni hypo- or hyper-biofilm mutant identified to date exhibits planktonic growth sensitivities and/or motility defects, as well as colonization and/or host cell interaction defects (14, 18, 20, 39, 40, 57, 74, 75, 80). Thus, the relevance of C. jejuni biofilms to colonization might be addressable only via studies of mutant strains in which altered biofilm formation is the only observable difference from the wild type.

Collectively, this work has yielded novel insight into the importance of the C. jejuni LOS in a number of pathogenesis-associated properties. Analysis of our truncation series also identified the relevance of specific LOS moieties and their respective transferases to specific aspects of stress survival and/or pathogenicity. This study further highlights the dynamic nature of the C. jejuni cell envelope and, through use of a mouse competition model, provides the first direct evidence for a role of the LOS in colonization. Future work stemming from this platform should lend even more insight into the pathogenesis of this important food-borne organism.

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