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A Deep-Rough Mutant of *Campylobacter jejuni* 81-176 Is Noninvasive for Intestinal Epithelial Cells

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A *waaF* mutant of *Campylobacter jejuni* 81-176 showed decreased invasion of INT407 cells in vitro and increased sensitivity to some antibiotics compared to what was seen with the wild-type strain.

The food-borne pathogen *Campylobacter jejuni* is one of the principal causes of human gastroenteritis worldwide (7, 17). In addition, *C. jejuni* is associated with the development of a devastating neurological disorder, Guillain-Barré syndrome, perhaps because of molecular mimicry between the outer core of *Campylobacter* lipooligosaccharide (LOS) and the carbohydrate moiety of human gangliosides (15, 16). *C. jejuni* strain 81-176 has been shown in two human volunteer feeding studies to cause diarrhea (5) and has been studied extensively for molecular mechanisms of pathogenesis (2, 3, 5, 12–14, 20). *C. jejuni* 81-176 LOS (Fig. 1) is composed of two covalently linked domains: lipid A, a hydrophobic anchor, and a nonrepeating core oligosaccharide consisting of an inner and an outer core (1, 10). The carbohydrate structure of the outer core, consisting of hexoses and *N*-acetylglucosamine, primarily mimics GM_2 and GM_3 gangliosides, with minor amounts of GD_2 and GD_{1b} mimics being detectable (10). Two outer-core mutants have been assayed for their ability to invade human INT407 cells (10). A mutant lacking *N*-acetylneuraminic acid invaded INT407 cells at the same levels as did the wild-type strain, and interestingly, a mutant lacking the terminal GalNAc showed a modest, but statistically significant, increase in ability to invade INT407 cells (10).

The inner-core region of *C. jejuni* 81-176 LOS is structurally similar to the same region of lipopolysaccharide (LPS) in enteric bacteria such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (11) (Fig. 1). It consists of a single 3-deoxy-D-manno-2-octulosonic acid residue (Kdo) attached to lipid A and two L-glycero-D-manno-heptose (heptose) residues attached to the Kdo residue. The inner-core region also contains a glucose residue and phosphoethanolamine moiety attached to the first heptose residue. To better understand the relationship between biosynthesis of the LOS and pathogenesis, we initiated a study of the inner-core oligosaccharide of LOS from *Campylobacter* strain 81-176. The *waaF* gene was identified in an ordered partial Sau3A-digested genomic library of 81-176 DNA cloned into λ-ZAPII (L. C. Holder and P. Guerry, unpublished data). Four open reading frames (ORFs), organized in a fashion similar to what is seen in other *C. jejuni* strains (8), were revealed from the sequence analysis of plasmid pLCH3-4 (Fig. 2). ORF2 encoded a protein of 318 amino acids with a predicted molecular mass of 36 kDa that showed significant similarity to several WaaF enzymes that catalyze the transfer of the second heptose to the core oligosaccharide of LPS and LOS. This ORF product was 88% identical to the recently reported WaaF protein from *C. jejuni* 11828 (19). In addition, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by silver staining showed that the 81-176 *waaF* gene is able to restore LPS assembly by complementation of a *Salmonella* serovar Typhimurium *waaF511* mutant strain, SL3789 (22) (data not shown).

A *waaF*::Cm insertional mutant was generated as previously described (9). One insertion that occurred 146 bp from the translational start of *waaF* was selected to electroporate 81-176 to Cm^r, and a double crossover was confirmed by PCR (data not shown). The LOS from the *waaF*::Cm mutant migrated more quickly than that of the wild-type strain (Fig. 3, lane 2 versus 1). The presence of an LOS similar to that of the wild type was restored when the *waaF* mutant strain was complemented in *trans* with pLCH1 (Fig. 2), which contains the 81-176 *waaF* gene (Fig. 3, lane 3). Table 1 lists the strains and plasmids used in this study.

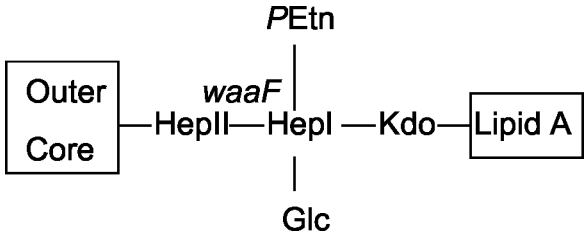

FIG. 1. Schematic showing the molecular structure of the 81-176 LOS and highlighting the inner-core region. Hep, heptose; *P*Etn, phosphoethanolamine. The *waaF* gene encodes heptosyltransferase II, which is involved in the addition of the second heptose as indicated.

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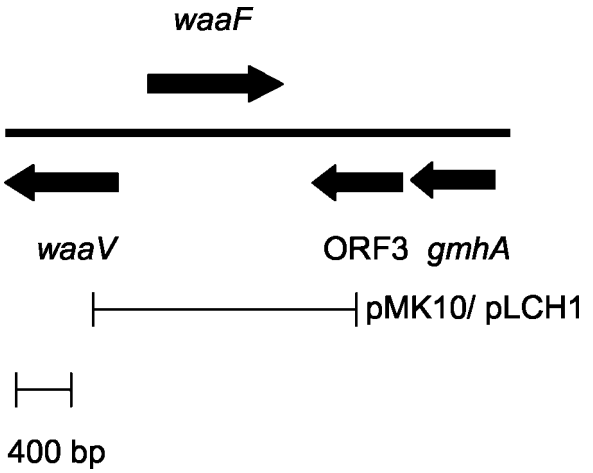

FIG. 2. Schematic representation of the *C. jejuni* 81-176 genes identified in the insert in plasmid pLCH3-4. The region of DNA that contained the 81-176 *waaF* gene (ORF2) was cloned into PCR 2.1 TOPO (Invitrogen, Carlsbad, Calif.) to generate pMK10 and into the kanamycin-resistant *Campylobacter* shuttle plasmid pRY107 (24) to generate pLCH1 for complementation studies in *Salmonella* serovar Typhimurium and *C. jejuni waaF* mutants, respectively. The ORF designated *waaV* encoded a protein that was 91% identical to WaaV (Cj1146c) from the genomic strain of *C. jejuni*, NCTC 11168. ORF3 encoded a protein that was 100% identical to an unpublished sequence from another strain of *C. jejuni* (NCTC 11351; accession number AAK08966) but was not present in NCTC 11168. GmhA of strain 81-176 was 97% identical to the corresponding protein from NCTC 11168 (Cj1149c).

A previous study has shown that the permethylated core oligosaccharide of the parental strain 81-176 possesses pseudomolecular ions with *m/z* values of 1,943, 2,188, 2,548, and 2,752 $[M + H]^+$ corresponding to cores exhibiting mimicry of GM_3, GM_2, GD_2, and GD_{1b} gangliosides, respectively (10). In contrast, the mass spectrum of the truncated LOS species in the *waaF* mutant possessed a pseudomolecular ion with an *m/z* value of 926 $[M + H]^+$ and related daughter ions that may be interpreted as those of an LOS species that contains glucose, heptose, phosphoethanolamine, and Kdo (Fig. 4). This result supports the loss of the second heptose residue from the LOS. Furthermore, glycosylation of a complete LOS requires the transfer of the sugar catalyzed by the heptosyltransferase II, WaaF.

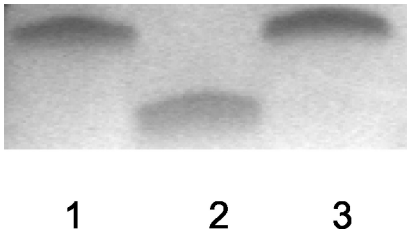

FIG. 3. Comparison of LOS from *C. jejuni* 81-176 with that from the *waaF* mutant. Proteinase K-digested whole-cell preparations were electrophoresed on 16% Tricine gels and silver stained. Lane 1, 81-176; lane 2, *waaF* mutant; lane 3, *waaF* mutant complemented in *trans* (pLCH1).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant property or characteristic(s)	Reference or source
C. jejuni strains		
81-176	Penner serotype 23, 36	5, 14
81-176*waaF*::*cat*	Nonpolar insertion in *waaF*, Cm^r	This study
Serovar Typhimurium strains		
SL3770	*waa*$^+$	SGSC[a]
SL3789	*waaF*511	SGSC
E. coli strain		
DH5α	F^- ϕ80d *lacZ* ΔM15	Invitrogen
Plasmids		
pCR2.1	Cloning vector, Ap^r	Invitrogen
pMK10	pCR2.1 with a 1.5-kb PCR-derived insert containing *C. jejuni* 81-176 *waaF*, Ap^r	This study
pRY107	*E. coli-C. jejuni* shuttle vector, Km^r	24
pLCH1	pRY107 with a 1.5-kb XbaI-BamHI fragment containing *waaF* from pMK10, Cm^r Km^r	This study

[a] SGSC, Salmonella Genetic Stock Centre.

Since there was no difference between the wild-type and *waaF* mutant strains in growth or motility (data not shown), the ability of the *C. jejuni* 81-176 deep-rough mutant to invade INT407 human intestinal epithelial cells was examined as previously described (2, 3, 18, 25). The data shown in Fig. 5 are expressed as the percentage of the input inoculum internalized during the 2-h gentamicin kill assay. The level of wild-type 81-176 invasion (1.238% ± 0.634%) was 14-fold higher than that of the *waaF* mutant (0.08% ± 0.074%). When the *waaF* mutant was complemented in *trans* with pLCH1, the level of invasion was restored to wild-type levels (2.0% ± 1.41%).

Deep-rough mutants of enteric bacteria are hypersensitive to hydrophobic antibiotics and detergents due to major alterations in the outer membrane (11, 21). As shown in Table 2, the *C. jejuni waaF* mutant showed hypersensitivity to the

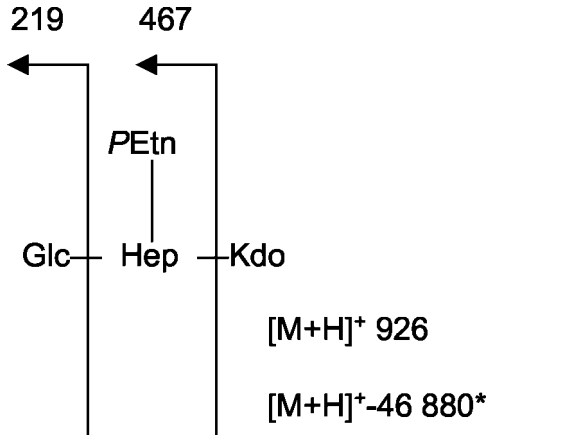

FIG. 4. Analysis of the positive-ion fast atom bombardment mass spectrometry of the permethylated core oligosaccharide from LOS of 81-176 *waaF*. Numbers refer to *m/z* values for ions. The asterisk indicates that derivatization resulted in partial degradation of the Kdo with a loss of 46 mass units.

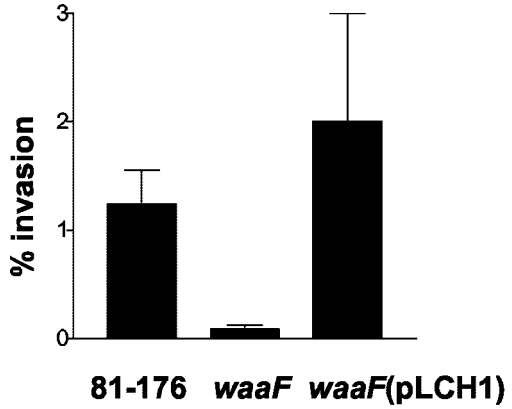

FIG. 5. Comparison of levels of invasion of INT407 cells. Invasion levels are shown as percentages of the input inoculum internalized during a 2-h invasion assay. Data shown are the mean results from two invasion assays for the complemented mutant and from four invasion assays for the wild type and the *waaF* mutant.

antibiotics fusidic acid and novobiocin and the detergent SDS compared to what was seen with the wild type. However, neither the wild-type nor the *waaF* mutant strain was sensitive to bacitracin, and there was no difference in the sensitivities to gentamicin, which was used in the invasion assays. Wild-type levels of resistance to fusidic acid, novobiocin, and SDS were restored when the *waaF* mutant was complemented in *trans*.

Although a *waaF* mutant (19) of another *C. jejuni* strain with a core structure distinct from that of 81-176 (10) has been described, this is the first report of both the structure of a *waaF* core and complementation in *trans* and the first report to examine the effect of deep-rough mutations on virulence. *waaF* mutants of a number of gram-negative bacteria have been studied (4, 6, 22), and *Haemophilus ducreyi* and *Neisseria gonorrhoeae waaF* mutants have been shown to be less virulent (4, 23). This reduction in invasion by the *C. jejuni waaF* mutant may reflect the need for a complete LOS for proper membrane function. As with other deep-rough mutants, mutation of *waaF* led to hypersensitivity to various antibiotics. We are in the process of determining whether loss of WaaF activity may result in additional changes in the composition or structure of the outer membrane.

Nucleotide sequence accession number. The nucleotide sequence of the region of the chromosome of *C. jejuni* 81-176 presented in Fig. 2 has been deposited in GenBank under accession number AY423899.

TABLE 2. Sensitivity to antibiotics and SDS

Strain	Zone diam (mm) with agent[a]:			
	Novobiocin	Bacitracin	Fusidic acid	SDS
81-176	11	7	10	14
waaF mutant	16	7	15	18
waaF(pMK10)	13.5	7	10	11

[a] Compounds were impregnated onto 7-mm-diameter disks. A zone size of 7 mm is equal to the diameter of the disk and corresponds to complete resistance.

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