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The role of WlaRG, WlaTB and WlaTC in lipooligosaccharide synthesis by *Campylobacter jejuni* strain 81116

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ABSTRACT

Campylobacter jejuni is a major bacterial cause of gastroenteritis world-wide. *C. jejuni* produces a range of glycans including lipooligosaccharide (LOS), an important virulence factor. The genetic content of the LOS synthesis locus varies between *C. jejuni* strains and 19 classes have been described. Three LOS synthesis genes of *C. jejuni* strain 81116 (NCTC 11828), *wlaRG*, *wlaTB* and *wlaTC* were the focus of this study. WlaRG and the remaining two proteins of interest share sequence similarity to aminotransferases and glycosyltransferases, respectively. These genes were insertionally inactivated and phenotypically characterised. Each mutant produced truncated LOS. Mutants lacking WlaRG, WlaTB and WlaTC produced LOS with reduced immunogenicity. Both the *wlaRG* and *wlaTC* mutants were non-motile and aflagellate. *In vitro* invasion and adhesion assays revealed that the *wlaRG*, *wlaTB* and *wlaTC* mutants displayed reduced adherence to chicken embryo fibroblasts. All mutants were less invasive of human cells than 81116 confirming the role of intact LOS during invasion of human cells *in vitro*. Here we propose the general composition for the 81116 LOS core backbone based on capillary electrophoresis-mass spectrometry.

1. Introduction

Campylobacter jejuni is a major bacterial cause of human gastroenteritis in both developed and developing nations. It is estimated that *C. jejuni* is responsible for approximately 90% of human *Campylobacter* spp. enteritis cases [1]. *C. jejuni* infection is usually attributed to the consumption of or contact with contaminated food or water, especially poultry [2,3]. *Campylobacter* spp. enteritis is usually self-limiting and characterised by severe abdominal pain, diarrhoea and can be accompanied by fever and vomiting [4]. Despite the advances made in our understanding of *C. jejuni* pathogenesis, *Campylobacter* spp. enteritis remains a serious public health burden and specific treatment and preventative strategies have not yet been developed.

Surface polysaccharides (PS) are known virulence factors important to the pathogenesis of *C. jejuni* [5–8]. *C. jejuni* produces lipooligosaccharide (LOS), capsular polysaccharide (CPS) [9,10] and many glycosylated surface proteins [11], including flagellin with *O*-linked glycans and multiple proteins with an *N*-linked glycan [12]. The glycome of *C. jejuni* was the subject of a comprehensive review article [13]. LOS consists of lipid A and branched-chain oligosaccharide core with an inner more conserved region and an outer more variable region. The core is linked via a 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid (Kdo) residue to the endotoxic lipid A moiety which is anchored into the outer leaflet of the outer membrane [14–16]. The lipid A consists of a glucosamine backbone and variations in the phosphorylation of lipid A or length of the fatty acid chain may affect the host immune response [17]. The only PS investigation published on *C. jejuni* 81116 describes an "LPS-like" PS which contains glucose (Glc), galactose (Gal) and *N*–acetyl-D-glucosamine (GlcNAc) [18].

LOSs are important virulence factors involved in serum resistance, resistance to phagocytic killing, adhesion to and invasion of host cells, are endotoxic and undergo phase variation [6,19–22]. LOSs from certain strains of *C. jejuni*, in particular those strains which produce LOS containing sialic acid residues, have been linked

Abbreviations: aa, amino acid; CE-MS, capillary electrophoresis-mass spectrometry; CPS, capsular polysaccharide; ddHexN, deoxyhexosamine; DMEM, Dulbecco's modified Eagle's media; ESI, electrospray ionisation; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetyl-D-glucosamine; Hep, heptose; Hex, hexose; HexNAc, *N*-acetyl hexosamine; kan^R, kanamycin resistance gene; Kdo, 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid; LOS, lipooligosaccharide; PS, polysaccharide; TEM, transmission electron microscopy; Res, residue.

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to the development of neurological complications [23,24]. Sialic acid residues are also found on human gangliosides and it is believed that molecular mimicry is involved in *C. jejuni*-associated Guillain-Barré syndrome and Miller Fisher syndrome [24–26].

The genes involved in the biosynthesis of each type of PS are mostly organised into clusters [27,28], the LOS cluster [29], the *kps* cluster encoding capsule biosynthesis genes [9] and the *pgl* cluster involved in general protein glycosylation [11]. The genetic content of the LOS biosynthesis cluster varies between *C. jejuni* strains and consequently so too do the core structures of the LOS molecules produced [30]. The LOS structures of *C. jejuni* have been classified into 19 classes [30–32].

The DNA sequence analysis of the LOS cluster (*wla*II) from *C. jejuni* 81116 was performed and published by Oldfield and colleagues in 2002 for the region from *rlmB* to *waaF*, GenBank accession number AF343914 [29]. The complete LOS cluster from *waaC* to *waaF* is now available from the genome sequence, GenBank accession number CP000814 [33]. It is a class E locus [30] and contains 19 genes (Fig. 1) when including *waaC* and *waaF* that encode the heptosyltransferases I and II, respectively. For the purpose of this study the *wla* nomenclature proposed by Reeves and colleagues [34] will be used for individual genes located within the LOS synthesis locus of *C. jejuni* strain 81116 except for genes already given names that reflect their function and appear elsewhere in the literature (Supplementary Table 1). Three genes located within the LOS locus of *C. jejuni* strain 81116, *wlaRG*, *wlaTB* and *wlaTC* (Fig. 1), were the focus of this study. Other genes in the LOS locus of *C. jejuni* 81116 have been investigated by colleagues in our research group, but have not yet been published. Phenotypic characterisation of the three knock-out mutants insertionally inactivated with a kanamycin resistance gene (kan^R), Δ*wlaRG*::kan^R, Δ*wlaTB*::kan^R and Δ*wlaTC*::kan^R confirms their involvement in LOS biosynthesis. Interestingly, inactivation of both *wlaRG* and *wlaTC* resulted in non-motile and aflagellate mutants. This may suggest that the proteins produced by each of these genes are also involved in flagellin glycosylation which, in turn, appears to be required for flagella construction. We also propose the general composition backbone of *C. jejuni* 81116 core LOS.

2. Results

2.1. Deduced aa sequence analysis

Blastp alignment of the deduced aa sequences from each of the genes of interest identified proteins of similar sequences, including some of known functions (Table 2). Sequence analysis summarised in Table 2 suggests that WlaTB and WlaTC are most similar to glycosyltransferases and WlaRG to aminotransferases. WlaRG contains a conserved domain which places it within the DegT/DnrJ/EryC1/StrS aminotransferase family (accession no. pf01041) and is likely to be a pyridoxal-phosphate dependent aminotransferase. WlaTB shares aa sequence similarity with CPS biosynthesis proteins (Table 2). The aa sequence of WlaTC showed the greatest similarity to HtrL (synonym YibB) an uncharacterised protein found in *Escherichia coli* K12, *E. coli* O157:H7 and *Shigella flexneri* (Table 2). WlaTC did not share sequence similarity with any proteins of known function

2.2. Each knock-out mutant was generated from the C. jejuni 81116 parent strain by insertional inactivation using the kan^R gene as a selective marker

The orientation of the kan^R gene was determined for each mutant by PCR-RFLP. Both the Δ*wlaRG*::kan^R and Δ*wlaTC*::kan^R mutants contained the kan^R gene in the opposite orientation to the gene of interest. The mutants Δ*wlaTB*::kan^R contained the kan^R gene in the same orientation as the gene of interest.

2.3. Phenotypic characterisation of three mutants each lacking WlaRG, WlaTB or WlaTC

Each mutant was created in *C. jejuni* strain 81116 and phenotypically characterised. SDS-PAGE showed that the LOS of the wild type LOS is migrating just below the 16 kDa protein marker while the LOS molecules produced by each of the three mutants were truncated when compared to the parent strain (Fig. 2a). Mass spectrometry was used to determine the precise mass difference between the LOS molecules of the parent strain and of the mutants (see text below and Supplementary Table 2). The size of the CPS produced by the mutants did not appear to be altered by the mutation, when compared to the parent strain CPS (Fig. 2b). The LOSs produced by each mutant was less reactive with the anti-LOS antisera than the parent strain LOS (Fig. 2c). The parent strain and mutant Δ*wlaTB*::kan^R are motile. However, motility was lost in both Δ*wlaRG*::kan^R and Δ*wlaTC*::kan^R mutants (Fig. 3). The non-motile mutants were confirmed as being aflagellate by TEM while the motile mutants expressed intact flagella (Fig. 4).

The capacity for each mutant to adhere to and invade human intestinal epithelial (INT407) and chicken embryo fibroblasts (CEF-DF1) cell lines was determined (Fig. 5). Mutants Δ*wlaRG*::kan^R, Δ*wlaTB*::kan^R and Δ*wlaTC*::kan^R were less adherent to CEF-DF1 cells than the parent strain, $p = 0.0019$, 0.0011 and 0.0016 respectively. The parent strain was more adherent to CEF-DF1 cells than to INT407 cells, $p = 0.0023$. The Δ*wlaTB*::kan^R mutant was less invasive of CEF-DF1 cells than the parent strain, $p = 0.0431$. The parent strain was more invasive in the human cell line than all three mutants, $p = 0.0075$ (Δ*wlaRG*::kan^R), $p = 0.0071$ (Δ*wlaTB*::kan^R) and $p = 0.0075$ (Δ*wlaTC*::kan^R). The parent strain was significantly more invasive in the INT407 cell line than in the CEF-DF1 cell line, $p = 0.0079$.

Preliminary GC-MS analysis of the LOS monosaccharides present in each mutant and parent strain suggests that the LOSs produced by mutant Δ*wlaTB*::kan^R contains less GlcNAc. GlcNAc compositions expressed as a percentage of total sugars for all strains are 51.6% (parent strain), 38.1% (Δ*wlaRG*::kan^R), 46.3% (Δ*wlaTC*::kan^R) and 21.2% (Δ*wlaTB*::kan^R) based on GC-MS peak

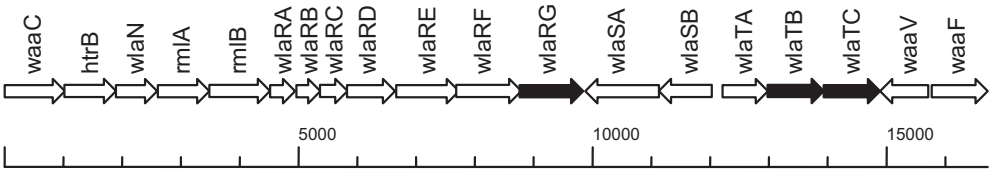

Fig. 1. A schematic diagram of the LOS synthesis locus from *C. jejuni* strain 81116, GenBank accession number CP000814 (16,718 bp) [33]. Black arrows indicate insertionally inactivated genes phenotypically characterised in this study. White arrows indicate genes that were not investigated in this study. The gene nomenclature used is the system proposed by Reeves and colleagues [34] and is described in more detail in Supplementary Table 1.