The Sialylated Lipooligosaccharide Outer Core in Campylobacter jejuni
Is an Important Determinant for Epithelial Cell Invasion\textsuperscript{\textdagger}

Rogier Louwen,1* Astrid Heikema,1 Alex van Belkum,1 Alewijn Ott,1† Michel Gilbert,3 Wim Ang,1‡ Hubert P. Endtz,4 Mathijs P. Bergman,1,‡ and Edward E. Nieuwenhuis2

Departments of Medical Microbiology and Infectious Diseases1 and Pediatrics;2 Erasmus MC, Rotterdam, The Netherlands, and Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada3

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\textit{Campylobacter jejuni} is a frequent cause of bacterial gastroenteritis worldwide. Lipooligosaccharide (LOS) has been identified as an important virulence factor that may play a role in microbial adhesion and invasion. Here we specifically address the question of whether LOS sialylation affects the interaction of \textit{C. jejuni} with human epithelial cells. For this purpose, 14 strains associated with Guillain-Barré syndrome (GBS), 34 enteritis-associated strains, the 81-176 reference strain, and 6 Penner serotype strains were tested for invasion of two epithelial cell lines. \textit{C. jejuni} strains expressing sialylated LOS (classes A, B, and C) invaded cells significantly more frequently than strains expressing nonsialylated LOS (classes D and E) (\(P < 0.0001\)). To further explore this observation, we inactivated the LOS sialyltransferase (Cst-II) via knockout mutagenesis in three GBS-associated \textit{C. jejuni} strains expressing sialylated LOS (GB2, GB11, and GB19). All knockout strains displayed significantly lower levels of invasion than the respective wild types. Complementation of a \textit{Δcst-II} mutant strain restored LOS sialylation and reset the invasiveness to wild-type levels. Finally, formalin-fixed wild-type strains GB2, GB11 and GB19, but not the isogenic \textit{Δcst-II} mutants that lack sialic acid, were able to inhibit epithelial invasion by viable GB2, GB11, and GB19 strains. We conclude that sialylation of the LOS outer core contributes significantly to epithelial invasion by \textit{C. jejuni} and may thus play a role in subsequent postinfectious pathologies.

\textit{Campylobacter jejuni} is recognized as a leading cause of bacterial gastroenteritis worldwide. Poorly handled or improperly cooked poultry meat, raw milk, pets, and untreated water are thought to be sources of infection (28). The disease spectrum caused by \textit{C. jejuni} ranges from asymptomatic infection to severe inflammatory bloody diarrhea (19). Furthermore, \textit{C. jejuni} infection has been associated with the development of postinfectious complications such as the Guillain-Barré syndrome (GBS) (26). The apparent variation in gastrointestinal disease outcome is likely to be affected by the expression of virulence factors that are associated with specific pathogenic mechanisms, e.g., \textit{C. jejuni} motility (24), attachment (18), and invasion (6, 16, 38). Motility and chemotaxis appear to be necessary for the epithelial adherence of \textit{C. jejuni}, whereas the expression of functional flagella may determine the capacities of \textit{C. jejuni} to invade the epithelium and to effectively colonize the mouse intestine (38, 41, 44, 45).

Next to the role of flagella in the regulation of \textit{C. jejuni} invasiveness, lipooligosaccharide (LOS) structures have generally been implicated in microbial invasion (15, 17, 21, 25, 33, 35, 37). To date, five major and distinctive LOS biosynthesis gene clusters, referred to here as LOS classes, have been described for \textit{C. jejuni} (31), and this number continues to increase (30). Sequencing and microarray analysis of the LOS biosynthesis gene locus of the \textit{C. jejuni} genome have also revealed this locus to be highly variable (11, 15), which may contribute to the variation in \textit{C. jejuni}-associated pathologies. Furthermore, it has been shown that \textit{C. jejuni} strains may also acquire these LOS synthesis genes from other \textit{C. jejuni} strains by means of horizontal exchange (10, 34).

A subgroup of \textit{C. jejuni} strains that express the LOS class A, B, or C gene locus harbor genes involved in sialic acid biosynthesis and are therefore able to synthesize sialylated LOS (9, 11, 12, 14). The \textit{cst-II} gene encodes a sialyltransferase (7) that is necessary for the transfer of sialic acid onto the LOS core in \textit{C. jejuni} class A and B strains. \textit{C. jejuni} class C strains depend on the \textit{cst-III} gene for LOS sialylation. Hence, only \textit{C. jejuni} strains expressing LOS class A, B, or C are capable of LOS sialylation. Previously, we have shown that the presence and expression of the \textit{cst-II} gene is specifically associated with GBS and is required for the induction of antiganglioside antibody responses, which are the hallmark of this postinfectious complication (12, 39). Based on this prior work, we hypothesized that LOS sialylation (and consequently \textit{C. jejuni} LOS subclasses) may be involved in \textit{C. jejuni} invasiveness.

Therefore, a panel of 48 human isolates and 7 human control strains was assessed for invasiveness for two human epithelial carcinoma cell lines (Caco-2 and T84). To specifically explore the role of sialylation, we generated three GBS-associated sialyltransferase (Cst-II) knockout \textit{C. jejuni} strains (GB2 \textit{Δcst-II}, GB11 \textit{Δcst-II}, and GB19 \textit{Δcst-II}). These GB2 \textit{Δcst-II}, GB11 \textit{Δcst-II}, and GB19 \textit{Δcst-II} mutants were tested for their abilities to adhere to and invade Caco-2 cells. Finally, we
investigated whether complementation of the \( \Delta \text{cst-II} \) mutant would restore the invasion-associated function of this gene product.

**MATERIALS AND METHODS**

**Bacterial strains.** Fourteen GBS- and 34 enteritis-associated \( C. \text{jejuni} \) strains isolated from Dutch patients, 6 Penner serotype strains, and the 51-176 enteritis reference strain were used in this study (see Table 1). To minimize in vitro passages, \( C. \text{jejuni} \) strains were recovered from the original patient-isolated glycerol stock by culturing on Butzler agar plates (Becton Dickinson, Breda, The Netherlands). A second passage was allowed for optimal vitality before these strains were used in experiments. After recovery, cells were harvested in Hanks balanced salt solution (Life Technology, Breda, The Netherlands), and densities were adjusted according to the optical density at 600 nm (OD600).

**Typing of the LOS biosynthesis gene cluster.** To determine the class of LOS locus present in each \( C. \text{jejuni} \) strain, genomic DNA was isolated using the DNeasy tissue kit (Qiagen, Venlo, The Netherlands). PCR analysis was done with primer sets specific for classes A, B, C, D, and E as previously described (12). PCR assays were performed in a Perkin-Elmer GeneAmp PCR system, model 9700 (Applied Biosystems, Nieuwkerk aan de IJssel, The Netherlands), with 25 cycles of 94°C, 1 min at 52°C, and 2 min at 72°C.

**Knockout mutagenesis.** Strains GB2 and GB11 and their \( \Delta \text{cst-II} \) mutants, GB2-\( \Delta \text{cst-II} \) and GB11-\( \Delta \text{cst-II} \), respectively, have been described previously (12). A \( \Delta \text{cst-II} \) mutant of a third GBS-related strain that is described here, GB19, was generated by the same procedure that was used for the knockout mutagenesis of strains GB2 and GB11 (12). Briefly, the target gene (\( \text{cst-II} \)) and approximately 700 bp of upstream and downstream flanking sequences were amplified and cloned into the pGem-T Easy vector (Promega Corp., Leiden, The Netherlands). Inverse PCR was used to introduce a BamHI restriction site and a deletion of approximately 800 bp in the target gene. Inverse PCR products were digested with BamHI (Fermentas, St. Leon-Rot, Germany) and ligated to the BamHI-digested chloramphenicol resistance (Cmr) cassette. Constructs were electroporated into electrocompetent GB19 \( C. \text{jejuni} \) cells, and recombinants were selected on Mueller-Hinton plates (Becton Dickinson, Breda, The Netherlands) containing 20 \( \mu \)g/ml chloramphenicol (Difco, Alphen aan den Rijn, The Netherlands) and were incubated at 42°C under a microaerobic environment.

**Mass spectrometry.** Samples were prepared for LOS mass spectrometric analysis by overnight growth of \( C. \text{jejuni} \) strains at 37°C on Butzler agar plates under a microaerobic atmosphere. Material from one confluent agar plate under a microaerobic atmosphere was harvested and treated with proteinase K at 60 \( \mu \)g/ml, RNase A at 200 \( \mu \)g/ml, and DNase I at 100 \( \mu \)g/ml (Promega, Leiden, The Netherlands). O-deacetylated LOS samples were prepared and analyzed by capillary electrophoresis coupled to electrospray ionization mass spectrometry (23).

**Implementation of the cst-II gene.** We used site-specific homologous recombination to restore the wild-type phenotype of the GB11-\( \Delta \text{cst-II} \) mutant strain (unpublished data). Briefly, a construct containing the \( \text{cst-II} \) gene together with its promoter region and a gene encoding erythromycin resistance were cloned in its promoter region and a gene encoding erythromycin resistance were cloned in

**Inhibition of invasion.** Formalin fixed, wild-type \( C. \text{jejuni} \) strains and their \( \Delta \text{cst-II} \) mutants were used to inhibit invasion by viable \( C. \text{jejuni} \) GB2, GB11, and GB19. Briefly, GB2, GB11, GB19, and their \( \Delta \text{cst-II} \) mutants at a starting concentration of 5.0 \( \times \) 10^7 CFU/ml determined by the OD600, were fixed in 3.6% formalin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS for 10 min. The excess of formalin was removed by washing the fixed cells three times in PBS. The sterility of the control cultures confirmed that fixation was complete. Caco-2 cells at a density of 5.0 \( \times \) 10^5 per well were preincubated for 30 min with formalin-killed wild-type or \( \Delta \text{cst-II} \) mutant \( C. \text{jejuni} \) strains at a multiplicity of infection (MOI) ranging from 100 to 5,000. Subsequently, the Caco-2 cells were washed to remove excess dead \( C. \text{jejuni} \) bacteria, and then fresh medium was added. Viable wild-type cells were added at an MOI of 100, and invasion was assessed by the gentamicin exclusion protocol as described above.

**Statistical analysis.** Statistical analysis was performed using Instat software (version 2.05a; GraphPad Software, San Diego, CA). Because the invasiveness of strains differed widely, log transformation was used to equalize variances. Invasiveness was expressed as the geometric mean number of CFU per milliliter retrieved from the infected cell line in all three to six invasion experiments performed per \( C. \text{jejuni} \) strain. Differences in invasiveness between LOS class A, B, and C strains and LOS class D and E strains, and between GBS-associated and enteritis-associated strains, were tested for significance with a Mann-Whitney U test, since column statistics showed that the Gaussian distribution was unequal for the strains. A two-tailed value with \( P < 0.05 \) indicated statistical significance. Statistical analysis for differences in adherence and invasion between wild-type and knockout mutant strains was performed, and differences were tested for significance with a paired \( t \) test.

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RESULTS

LOS sialylation is associated with increased epithelial cell invasion. We observed a wide range of invasion capacities among the C. jejuni strains (Table 1). Categorization of C. jejuni strains into those carrying sialylated (n = 30) and nonsialylated (n = 18) LOS established that the sialylated-LOS producers, classes A, B, and C, were more invasive than the nonsialylated-LOS producers, classes D and E (median CFU...
These results are shown in Table 1. Carried out previously by immunological methods (1, 13). Strains (E98-623, 624, 652, 682, 706, 1033, and 1087) were sialylated for the GBS strains (GB2, GB3, GB4, GB11, GB13, GB19, and GB19) expressed sialylated LOS in the form of GD1c. In all three strains, knockout mutagenesis of cst-II resulted in loss of expression of sialylated LOS.

To exclude the possibility that differences in viability and growth rates would influence the results of our invasion assays, we assessed the growth rates of wild-type strains GB2, GB11, and their associated Δcst-II mutants. At an MOI of 100, wild-type and mutant strains adhered equally well to the human Caco-2 cell line (Fig. 3A). The only exception was the GB11 mutant, which displayed a lower level of adherence than wild-type strains GB2, GB11, and GB19 and their associated Δcst-II mutants are shown in Fig. 2. For a subset of strains, comprising GB3, GB4, GB13, GB17, GB22, GB23, GB25, and GB31, ganglioside mimic structures were determined previously by mass spectrometry (Table 1) (13). The LOS structures of the Penner serotype strains O:1, O:2, O:3, O:4, O:10, O:19, and 81-176 mimics were determined previously by mass spectrometry (Table 1) (13). The LOS structures of the Penner serotype strains O:1, O:2, O:3, O:4, O:10, O:19, and 81-176 have been characterized previously by other researchers (2–5, 15, 29). As can be seen by the absence of data for some strains in Table 1, mass spectrometry data on LOS structures were not available for all bacteria.

 Knockout mutagenesis of cst-II does not affect the bacterial growth rate significantly. To exclude the possibility that differences in viability and growth rates would influence the results of our invasion assays, we assessed the growth rates of wild-type strains GB2, GB11, and GB19 and their Δcst-II mutants in Mueller-Hinton medium and in the cell culture medium used in the Caco-2 cell invasion assays. No significant differences in growth rates were observed between the wild-type GB2, GB11, and GB19 strains and their Δcst-II mutants during the time span of our invasion experiments (data not shown).

Disruption of cst-II significantly affects the invasiveness of C. jejuni for intestinal epithelial cells. We compared the capacities of the C. jejuni wild-type strains GB2, GB11, and GB19 to adhere to and invade Caco-2 cells with those of their respective Δcst-II mutants. At an MOI of 100, wild-type and mutant strains adhered equally well to the human Caco-2 cell line (Fig. 3A). The only exception was the GB11 Δcst-II strain, which displayed a lower level of adherence than wild-type GB11 (P = 0.031). GB2 Δcst-II, GB11 Δcst-II, and GB19 Δcst-II all showed significant reductions in invasiveness relative to the wild-type strains.

FIG. 1. The invasiveness of C. jejuni is dependent on sialylation of the LOS. Scattergrams show the invasion of Caco-2 cells by Dutch C. jejuni strains, categorized with respect to the type of LOS that is expressed (sialylated LOS of classes A, B, and C [n = 30] versus nonsialylated LOS of classes D and E [n = 18]) (A) or the clinical outcome of infection, i.e., GBS (n = 14) versus uncomplicated gastroenteritis (n = 34) (B). Experiments were performed in triplicate and repeated at least three times. For each strain, a geometric mean outcome (number of CFU per milliliter) was calculated. The differences between the geometric means of groups of strains were tested with the Mann-Whitney U statistic. The median for each group of strains is shown.

FIG. 2. Proposed LOS outer core structures as determined by mass spectrometry analysis. Note that GB2 and GB11 express a mixture of the sialylated LOS ganglioside mimics GM1 and GD1a, whereas GB19 expresses sialylated LOS only in the form of GD1c. In all three strains, knockout mutagenesis of cst-II resulted in loss of expression of sialylated LOS.
to that of their wild-type parent strain ($P = 0.005$, $P = 0.002$, and $P = 0.008$, respectively) (Fig. 3B).

In order to study whether the role of sialic acid in \textit{C. jejuni} invasion is restricted to interactions with Caco-2 cells, a small selection of \textit{C. jejuni} strains (P3, GB2, GB11, and GB13) and $\Delta cst-II$ mutants (GB2 $\Delta cst-II$ and GB11 $\Delta cst-II$) were tested for invasiveness for the T84 human intestinal epithelial cell line (data not shown). The levels of invasiveness of all wild-type strains were similar in both cell types. Again, $\Delta cst-II$ mutants displayed reduced (by 1 to 1.5 log units) invasion of T84 cells. Together, these data establish that LOS sialylation contributes significantly to the invasion of intestinal epithelial cells by \textit{C. jejuni}. We excluded variation in microbial motility as the mechanism underlying the reduced invasion of the $\Delta cst-II$ mutant strains by performing quantitative swarming assays (data not shown).

Complementation of the GB11 $\Delta cst-II$ mutant restores expression of sialylated LOS. Site-specific homologous recombination was used to reinstall the\textit{cst-II} gene, together with its promoter region, in the GB11 $\Delta cst-II$ strain. Using HRP-labeled cholera toxin as a detection agent, we confirmed the expression of sialylated LOS of the wild-type GB11 strain and of three selected clones of the complemented GB11 $\Delta cst-II$ mutant by a Western blot assay (Fig. 4, lanes 1, 3, 4, and 5, respectively). The GB11 $\Delta cst-II$ mutant did not express sialylated LOS (Fig. 4, lane 2). LOS isolated from the 11168 genome strain was used as a positive control for the binding of the HRP-labeled cholera toxin (Fig. 4, lane 6).

Complementation of the GB11 $\Delta cst-II$ mutant restores invasiveness. The Western blot assay provided evidence that the complemented mutant was now capable of LOS sialylation. With the gentamicin exclusion assay, we were able to show that this complementation also restored invasiveness to wild-type levels (Fig. 5). These results reiterate the importance of LOS sialylation in invasion.

Fixed, sialylated LOS-containing strains inhibit invasion by their viable counterparts. The decreased invasiveness of GB2 $\Delta cst-II$, GB11 $\Delta cst-II$, and GB19 $\Delta cst-II$ and the restored wild-type invasion phenotype of the complemented GB11 $\Delta cst-II$ mutant clearly indicate a role for \textit{C. jejuni} LOS sialylation in invasion. In order to further address the involvement of LOS sialylation in invasion, we designed an inhibition assay. We preincubated the Caco-2 cells with formalin-fixed, nonviable sialylated wild-type strains (GB2, GB11, and GB19) before incubating the cells with viable sialylated wild-type strains (GB2, GB11, and GB19). We found reductions of as much as 1 to 2 log units in invasion by viable wild-type strains. When Caco-2 cells were preincubated with an excess of formalin-fixed nonsialylated LOS $\Delta cst-II$ mutants, no differences in invasion were found relative to the invasion control (Fig. 6). The control groups consisted of Caco-2 cells that were incubated only with the viable wild-type strain GB2, GB11, or GB19. These results corroborate that LOS sialylation is an important determinant of epithelial cell invasiveness.

**DISCUSSION**

The mucosal epithelial cells are the first to interact with enteric pathogens such as \textit{C. jejuni}. This microorganism may temporarily colonize the intestines in the absence of any clinical symptom. On the other hand, \textit{C. jejuni} has been implicated in the pathogenesis of immune-mediated pathologies, e.g., GBS. Because \textit{C. jejuni} infection can present with a such wide range of symptoms, it is crucial to further identify factors and mechanisms that control \textit{C. jejuni} epithelial invasion and persistence (42). We hypothesized that the factors that regulate \textit{C. jejuni} epithelial invasion may contribute directly to postinfectious sequelae, e.g., GBS.

Several \textit{C. jejuni} outer membrane proteins, e.g., CadF, JlpA, and PEB1, play roles in epithelial adhesion and invasion (8, 20, 32). Recently, PEB1 has also been identified as an amino acid transport system, which is essential for microbial growth (22). Previous studies that identified microbial LOS as a generally important factor for invasion have been confirmed for \textit{C. jejuni} (15, 17, 25, 33). Here we specifically addressed if and to what extent sialylation of \textit{C. jejuni} LOS contributes to microbial invasion. Therefore, we performed a large-scale survey by testing a heterogeneous panel of 48 human-isolated \textit{C. jejuni} strains, 7 human control strains, and 3 sialyltransferase (\textit{cst-II})
knockout strains. The knockout strains were previously shown to lack the capacity of LOS sialylation (12).

Our studies indicate that LOS sialylation facilitates epithelial invasion (Table 1), since C. jejuni strains expressing sialylated LOS invaded significantly more frequently than nonsialylated LOS strains ($P < 0.0001$). Two strains with presumed LOS sialylation displayed low invasiveness. These results show that LOS sialylation must be regarded as an important contributor to C. jejuni invasiveness but not the single determinant. Earlier reports support the hypothesis that several factors determine invasiveness (15, 17, 25, 33). Similar contributions of sialic acid to invasiveness have been established for other pathogens (36, 43). In contrast, one study reports on inhibition of invasion by sialic acid (40).

Our experiments with the GB2, GB11, and GB19 sialyltransferase (cst-II) knockout strains further established the importance of LOS sialylation, since these mutated strains expressing nonsialylated LOS displayed significantly lower invasiveness than their respective wild-type controls. The methods for generation of such knockout strains may be accompanied by various technical side effects, e.g., mutation of genes other than the target gene. Furthermore, insertion of an antibiotic resistance cassette may induce expression or silencing of adjacent genes and gene products. Therefore, we set up experiments using a complemented Δcst-II mutant strain. We show that this procedure indeed restored sialylation of the LOS (Fig. 4) and subsequent invasiveness to wild-type levels (Fig. 5).

In our studies, only the GB11 Δcst-II mutant strain showed diminished adherence relative to that of its wild-type parent strain, indicating a less important role for LOS sialylation in epithelial adhesion than in invasion. These findings indicate that adhesion and invasion are regulated by different sets of factors. Adhesion is likely established by proteins such as CadF, JlpA, and PEB1 (8, 20, 32), whereas invasion is more influenced by LOS sialylation in the strains we tested.

To support the hypothesis that invasion is facilitated by LOS sialylation, we established that formalin-fixed wild-type strains GB2, GB11, and GB19, but not the isogenic Δcst-II mutants, were able to inhibit epithelial invasion by viable GB2, GB11,

FIG. 4. Western blot assay for analysis of cholera toxin binding at the LOS of wild-type GB11, its Δcst-II mutant, and the complemented GB11 Δcst-II mutant strain. Lane 1, LOS of the GB11 wild-type strain; lane 2, LOS of the GB11 Δcst-II mutant strain; lanes 3, 4, and 5, LOS from three selected clones of the complemented GB11 Δcst-II mutant; lane 6, LOS of the 11168 genome strain, used as a positive control. The LOS band is present at around 7 kDa.

FIG. 5. Complementation of the GB11 Δcst-II mutant restores the wild-type phenotype for invasion observed with GB11. The C. jejuni wild-type strain GB11, the GB11 Δcst-II mutant, and the complemented GB11 Δcst-II (C) mutant were studied for invasion of human enterocyte-like Caco-2 cells. Data are geometric means from at least three independent experiments, each performed in duplo. Error bars, standard deviations.
and GB19 strains. These findings may have two implications. First, these data may help to identify novel epithelial invasion receptors. Second, these experiments may lead to the discovery of specific agents that can be used to block microbial invasion.

Previously, sialylation of \textit{C. jejuni} LOS was associated with \textit{C. jejuni} strains GB2, GB11, and GB19 invade Caco-2 cells via a sialylated-LOS-dependent mechanism(s). The levels of invasion by viable wild-type strains GB2 (A), GB11 (B), and GB19 (C) were assessed in the presence of either formalin-fixed GB2, GB11, or GB19 wild-type (wt) bacteria (sialylated LOS) or the respective fixed Δ\textit{cst-II} mutants (truncated LOS, nonsialylated). Data are means from at least three independent experiments; error bars, standard deviations.

In conclusion, we demonstrate that \textit{C. jejuni} strains expressing sialylated LOS have an overall increased capacity to invade intestinal epithelial cells. Knockout mutagenesis of the \textit{cst-II} gene and complementation and blocking experiments provide additional evidence on the role of LOS sialylation in the invasion of the intestinal epithelium. Understanding the function of LOS sialylation in epithelial cell invasion may provide us with potential target structures for future therapeutic interven-

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