Comparison of lipooligosaccharide biosynthesis genes of Campylobacter jejuni strains with varying abilities to colonize the chicken gut and to invade Caco-2 cells

Jens Müller, Birgit Meyer, Ingrid Hänel and Helmut Hotzel

Friedrich Loeffler Institute, Institute of Bacterial Infections and Zoonoses, Jena, Naumburger Str. 96a, 07743 Jena, Germany

INTRODUCTION

Campylobacter jejuni is the leading cause of bacterial foodborne diarrhoeal disease throughout the world (Blaser, 1997). The most common source of human infections is poultry products. The clinical spectrum of the disease ranges from asymptomatic infections to severe inflammatory bloody diarrhoea (Ketley, 1997). Furthermore, campylobacteriosis is also the most frequent antecedent infection before the onset of the post-infectious peripheral neuropathy Guillain–Barré syndrome (Nachamkin et al., 1998). Structural similarity between human gangliosides and C. jejuni lipooligosaccharides (LOSs), the so-called ganglioside mimicry, is thought to be involved in the elicitation of GBS and the related Miller–Fishersyndrome.

The completion of the sequencing of the genomes of C. jejuni NCTC 11168 and RM1221 has enabled the rapid identification of a large cluster of genes involved in LOS biosynthesis (Parkhill et al., 2000; Fouts et al., 2005). The LOS biosynthesis locus is one of the highly variable regions in the C. jejuni genome as indicated by the results of both DNA sequencing of these loci from different C. jejuni strains (Gilbert et al., 2002; Guerry et al., 2002) and microarray analysis (Dorrell et al., 2001; Leonard et al., 2004). The comparison of LOS biosynthesis loci from a variety of strains resulted in a grouping into eight LOS classes (A–H; Parker et al., 2005), based on the organization of about 40 distinct genes. The authors were able to classify a majority (80%) of the LOS biosynthesis loci from 123 C. jejuni strains utilizing PCR amplifications of genes from the cluster. The differences in the gene content of the LOS loci provide a basis for differences in LOS outer cores among C. jejuni strains. However, the same set of homologous genes is able to result in different LOS core structures. This is the result of multiple mechanisms that are used by C. jejuni to turn on or off a gene or to modulate the substrate specificities of the glycosyltransferases. Mechanisms responsible for the generation of variability in the LOS outer core structure include phase-variation because of homopolymeric tracts of DNA and gene inactivation by deletion or insertion of a single base.

While current research is focused on the role of LOS in the development of GBS, little attention has been placed on the
function of LOS structures in the pathogenesis of gastrointestinal disease. There is some evidence that LOS structures are important for the invasion of Campylobacter into epithelial cells. A site-specific insertional mutant in the cgtA gene of C. jejuni strain 81-176, which encodes an N-acetylgalactosaminyltransferase, showed a significant more than twofold increase in invasion of INT407 cells, compared to wild-type (Guerry et al., 2002). Variations in a homopolymeric tract of G-residues in the cgtA gene of C. jejuni strain 81-176 affected the length of the open reading frame, and these changes in cgtA expression were associated with changes of the LOS structure (Guerry et al., 2002). Similar phase variations and corresponding changes of LOS structure have been found in another LOS biosynthesis gene, wlaN of C. jejuni strain NCTC 11186 (Linton et al., 2000). We recently reported strain-specific differences in the occurrence of cgtB and wlaN genes, which both encode a β-1,3-galactosyltransferase, connected with different abilities to colonize the chick gut and to invade Caco-2 cells (Müller et al., 2006). However, cgtB was mostly detectable only in strains with strong colonization and invasion abilities but was absent in some colonizing strains.

In this study, we compared the presence of LOS biosynthesis genes from a panel of C. jejuni strains with different abilities to colonize the chick gut and to invade Caco-2 cells. We used PCR probing with specific primers and DNA sequencing to determine differences in LOS gene content. The aim of the study was to determine whether specific classes of genes are associated with the chicken colonization potential, invasive properties for human epithelial cells or animal reservoirs.

METHODS

Bacterial isolates. Forty C. jejuni isolates used in this study were of human, bovine or turkey origin. The strains were cultivated on Müller–Hinton blood agar at 37 °C under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) for 24 h. All isolates were characterized by their invasion ability determined in a Caco-2 cell culture model and their colonization ability determined in a chick model as described in previous studies (Hanel et al., 2004, 2007).

DNA preparation and PCR. Chromosomal DNA of the bacteria was extracted using the High Pure PCR Template Preparation kit (Roche Diagnostics). The target genes, primers and annealing temperatures are listed in Table 1. For the generation of PCR primers, the Primer Express software (Applied Biosystems) was used. PCR was performed in a DNA Thermal Cycler (Eppendorf) using standardized cycling parameters: 95 °C for 1 min for initial denaturation followed by 35 cycles of denaturation at 95 °C for 30 s, variable annealing temperature (Table 1) for 1 min, primer extension step at 72 °C for 1 min, and final extension step at 72 °C for 5 min. PCR products were analysed by electrophoresis on 1 or 2% agarose gels. DNA bands were stained with ethidium bromide and visualized under UV illumination using a Bio Imaging System (Syngene). The expected lengths of PCR products are shown in Table 1.

Sequencing of the wlaN gene. For sequencing of the wlaN gene homopolymeric tract, an approximately 435 bp intragenic fragment of the gene was amplified using genomic DNA and primer pair Cj1139cF and DL39 (Table 1). After purification of the expected bands with a QiAquick Gel Extraction kit (Qiagen), cycle sequencing reactions were carried out using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer’s instructions. Sequencing primers were Cj1139cF and DL39 (see above), and analysis was performed on an ABI 310 genetic analyser (Applied Biosystems).

Analysis of the transcription of cgtB in strain 972/96. Bacterial cells that had adhered to and invaded Caco-2 cells were recovered from an invasion assay (Hanel et al., 2004) after cell lysis by centrifugation and processed for RNA isolation using a RNeasy Protect Bacteria Mini kit (Qiagen) according to the manufacturer’s instructions. The concentration of RNA was determined by measuring the A260. One hundred and fifty nanograms of RNA was reverse-transcribed with random hexamer primers using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). A real-time PCR was carried out using qPCR MasterMix Plus for SYBR Green I (Eurogentec) and a MX 3000P real-time PCR system (Stratagene Europe). Primers for real-time PCR were cgtB-q-F (5′-GTG GAA AAA AAT TAT TGC AAA GAA-3′) and cgtB-q-R (5′-TCT AGT CTY TTA TCA CAC AAA GC-3′).

RESULTS AND DISCUSSION

An overview of the selected LOS genes and the PCR results in relation to colonization and invasion ability is shown in Table 2. In only 20% (3 of 15) of strains with no or weak colonization and invasion ability could the gene cgtB be detected (strains 972/96, 292/94 and 315/94), whereas it was found in 52% (13 of 25) of strongly invasive and colonizing strains. If one assumes that the β-1,3-galactosyltransferase encoded by cgtB (Gilbert et al., 2000) is a characteristic enzyme of strongly invasive strains, then the role of another β-1,3-galactosyltransferase encoded by wlaN (Cj1139c) should also be investigated. The overall level of amino acid identity between the two sequences is 58.4% (Linton et al., 2000). We found wlaN in 46.7% (7 of 15) of strains with no or weak colonization and invasion ability and in 60% (15 of 25) of strains with strong colonization and invasion ability. Linton et al. (2000) reported that the presence of an intragenic homopolymeric tract renders the expression of a functional wlaN gene product phase-variable, resulting in distinct cell populations with alternative LOS structures. Homopolymeric nucleotide tracts have been previously identified in the genome sequences of C. jejuni NCTC 11168 (Parkhill et al., 2000) and RM 1221 (Fouts et al., 2005). Investigations of several homopolymeric tracts in C. jejuni resulted in the hypothesis that the formation and/or selection of these tracts is not a random process, but is driven by as yet unknown mechanisms (Wassenaar et al., 2002). The polymorphism of these genes may be a mechanism by which C. jejuni survives selection bottlenecks between opportunities for growth within a host. In this study, an intragenic homopolymeric tract was found with six, eight or nine G-residues within wlaN by DNA sequencing (Table 2, Fig. 1). Only eight G-residues allow the transcription of a functional galactosyltransferase, whereas six or nine G-residues lead to two open reading frames
encoding two truncated hypothetical peptides. From the seven non- or weakly invasive strains with *wlaN* we found no strain with eight G-residues, six strains with six G-residues and one with nine G-residues. These results indicate that 80 % of strains with no or weak colonization and invasion ability have no functional β-1,3-galactosyltransferase encoded by *cgtB* or *wlaN*. However, we detected eight G-residues in the *wlaN* gene of four strongly invasive strains lacking *cgtB*. Our findings show that a functional β-1,3-galactosyltransferase encoded by *cgtB* or *wlaN* was detected in 68 % of strains with strong colonization and invasion ability.

Furthermore, in an analysis of transcriptional activity of *cgtB* in the non-invasive strain 972/96, we could not detect any mRNA of this gene by real-time PCR (data not shown). This indicates that unknown transcriptional regulation processes or defects in expression of *cgtB* could be a putative cause for the lack of colonization and invasion ability in this strain. After sequencing the *cgtB* gene of non- and weakly invasive strains 972/96, 292/94 and 315/94, we detected a single nucleotide exchange from G to A at position 528 in strain 972/96 leading to an amino acid change from arginine to lysine at position 176. In strain 292/94, we detected two single nucleotide changes which do not result in amino acid changes, and in strain 315/94, nucleotide changes were not detectable in the *cgtB* locus.

In eight strains with strong colonization and invasion ability we could not detect intact β-1,3-galactosyltransferase genes of *cgtB* and *wlaN* by PCR.

After complete sequencing of *C. jejuni* NCTC 11168, besides *wlaN* (Cj1139c) seven other genes encoding...
putative galactosyltransferases (wlaH, wlaG, Cj1136, Cj1138, Cj1434c, Gj1438c and Gj1440c) were annotated (Parkhill et al., 2000). Sequencing of C. jejuni RM1221 resulted in the annotation of two putative galactosyltransferases (CJE1278 and CJE1280) with no similarity to genes of NCTC 11168. PCR detection of all these gene loci in 40 C. jejuni isolates used in this study indicated no correlation between the occurrence of the genes and the colonization and invasion ability of the strains (data not shown). These putative galactosyltransferases were not able to compensate for the lack of cgtB and wlaN gene products in non-invasive strains.

Finally, the results suggest a correlation between the occurrence of a β-1,3-galactosyltransferase encoded by cgtB or wlaN in C. jejuni strains and their strong colonization and invasion ability in vivo and in vitro.
A UDP-glucose 4-epimerase of *C. jejuni* encoded by *galE* was shown to be involved in LOS synthesis and virulence (Fry et al., 2000). A *galE* deletion mutant of *C. jejuni* 81116 showed a reduction in its ability to adhere to and invade INT407 cells, whereas it was still able to colonize chickens to the same level as the wild-type strain. This is in contrast to our findings of a good correlation between colonization of the chick gut and ability of the strain to invade Caco-2 cells (Hänel et al. 2004). Nevertheless, we checked the 40 *C. jejuni* strains used in this study with respect to the occurrence of the *galE* gene by PCR. We detected this gene in all non- or weakly invasive strains as well as in nearly all strongly invasive strains (Table 2). Additionally, transcriptional analysis of *galE* in three invasive and three non-invasive strains showed no differences in transcription activity between the strains (data not shown). From these results, it can be concluded that *galE* is not responsible for the different colonization and invasion abilities of the investigated *C. jejuni* strains.

The *C. jejuni* LOS loci have been grouped into eight classes (A–H) based on gene content and organization utilizing PCR of genes from these loci (Parker et al., 2005). The detected LOS classes were not associated with the Penner serotypes but 14 of 16 GBS-associated isolates shared the same LOS class (A1). As *cgtB* and *wlaN* were among the genes used for the classification, a possible association of invasive and non-invasive strains with specific LOS classes was investigated. With additional primers targeting *neuB*-a, *cgtA2*, orf17d and orf19df (Table 1), a classification of the majority (65%) of LOS biosynthesis loci of 40 investigated *C. jejuni* strains became possible. In contrast to Parker *et al.* (2005) we found seven strains with concurrent detection of *cgtB* and *wlaN*. Furthermore, an association of invasive or non-invasive strains with a specific LOS class could not be detected.

An interesting correlation between LOS classes and source of the *C. jejuni* strains is shown in Table 3. Most of the human isolates possessed a class A1 or C locus whereas turkey isolates were positive for classes A2, C and unknown LOS classes. Most bovine isolates belonged to unknown LOS classes and a minority of them were positive for class C. These results are similar to the findings of Parker *et al.* (2005). LOS class C was detected in *C. jejuni* from all sources, suggesting a particular importance for the transmission of the bacteria from animals to humans. Moreover, classes A1 and B1 detected in turkey and human isolates are probably important for the transmission from poultry to humans. Further characterization of unknown LOS classes is necessary to determine the potential of bovine *C. jejuni* isolates to infect humans.

Another factor probably involved in colonization and invasion processes is *Campylobacter* protein glycosylation. *C. jejuni* 81-176 has been shown to contain a general protein glycosylation (pgl) system affecting many soluble and membrane-associated proteins (Szymanski *et al.*, 1999). Glycosylated flagellins of *Campylobacter* spp. have been characterized previously (Doig *et al.*, 1996; Thibault *et al.*, 2001). A *pglB* mutant of strain 81-176 encoding a putative oligosaccharyl transferase had reduced adhesion (38%) and invasion (4.4%) ability compared to the wild-type strain whereas growth rate and motility had not changed (Szymanski *et al.*, 2002). Furthermore, the glycoprotein VirB10 involved in a plasmid-encoded type IV secretion system could not be detected by Western blot analysis in the *pglB* mutant (Larsen *et al.*, 2004). In our study, we detected the *pglB* gene in all strains tested with one exception. The lack of *pglB* in strain 292/94 could be a reason for its weak colonization and invasion ability.

**Table 3.** Relationship between LOS classes and sources of the *C. jejuni* strains

<table>
<thead>
<tr>
<th>Source</th>
<th>A1</th>
<th>A2</th>
<th>B1</th>
<th>C</th>
<th>E</th>
<th>F</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>0</td>
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<tr>
<td>Human</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Bovine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>
However, in this strain we could not detect the Vir plasmid by PCR (data not shown). These results indicate additional functions of pgIB beside glycosylation of VirB10.

This study describes the putative involvement of glycosyltransferases in the pathogenicity of C. jejuni. Glycosyltransferases participate in different cellular processes with high variability such as LOS biosynthesis and protein glycosylation. LOS and glycosylated flagellins are structures of the outer surface of C. jejuni and are involved in interactions with the host cells. The variability of these outer structures seems to be an important virulence factor enabling the bacteria to overcome several bottlenecks of their surroundings. Homopolymeric tracts in single genes have been suggested as a specific mechanism to generate such variability. Nevertheless, the complete network from a single gene to structures determining the pathogenicity still remains unknown. Further work with specific mutants and gene expression studies has to be done.

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REFERENCES


