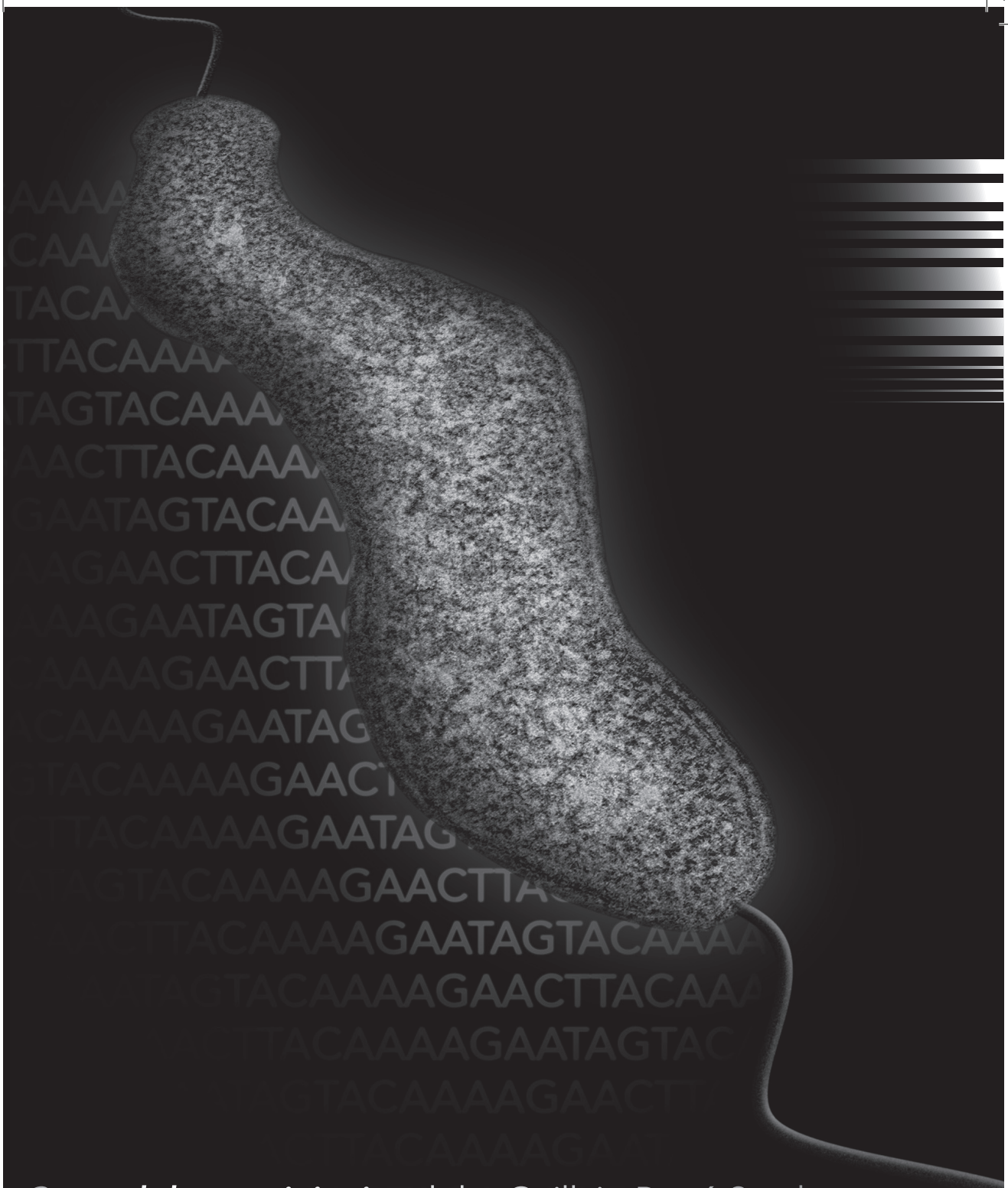

Campylobacter jejuni and the Guillain-Barré Syndrome: the role of bacterial genetic polymorphisms

Peggy Godschalk

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Campylobacter jejuni en het Guillain-Barré syndroom:
de rol van bacteriële genetische polymorfismen

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Campylobacter

Campylobacter spp. are small motile spiral-shaped or curved gram-negative bacteria. Organisms resembling campylobacters were observed for the first time in the colon of children who had died of "cholera infantum" by Theodor Escherich in 1886 (1). However, campylobacters were not recognized as common human enteric pathogens until the 1970s, when the development of selective growth media enabled diagnostic laboratories to perform routine stool cultures for *Campylobacter* (2).

Nowadays, infection with *Campylobacter* has been recognized worldwide as the most frequent cause of bacterial gastro-enteritis. The incidence of symptomatic *Campylobacter* enteritis in The Netherlands is estimated at 107.000 cases per year (3), whereas sero-epidemiological studies suggest that 2-3 million (a)symptomatic *Campylobacter* infections occur each year (4). The incidence of symptomatic infections is highest among young children (0-4 years of age), followed by young adults (15-29 years of age). In addition, there is a strong seasonality with a summer peak from June to September (3). Most *Campylobacter* infections are not related to outbreaks and occur as sporadic cases. Contact with and consumption of chickens is considered the main source for *Campylobacter* infection. Transmission from other animals (including pets and shellfish), contaminated water supplies and raw milk has also been described (5).

Although the genus *Campylobacter* comprises more than ten different species, the vast majority of *Campylobacter* infections in humans is caused by *C. jejuni*, followed by *C. coli*. After an incubation time of approximately 24-72 hours, the onset of symptoms is often abrupt with abdominal cramps followed by diarrhea. Fever is often present and bloody stools are observed in approximately 15%. The duration of symptoms varies; the diarrhea usually subsides after 2 to 3 days but abdominal pain or discomfort may persist for more than a week.

Campylobacter can be isolated from fecal specimens using selective media that inhibit growth of the normal gut flora. Most *Campylobacter* species require a microaerobic atmosphere and an incubation temperature of 37°C to 42°C for optimal growth. In most routine laboratories, the identification of *Campylobacter spp.* is based on growth on selective media, colony morphology, gram stain and a combination of biochemical tests. Several commercial assays are available to aid in the identification. In addition, multiple PCR assays for direct detection of *Campylobacter* in stool samples have been evaluated but are currently not frequently used in routine diagnostic procedures (6-8).

Treatment of presumed *Campylobacter* gastro-enteritis in otherwise healthy individuals is symptomatic and does not include antibiotics. Due to the increasing resistance against fluoroquinolones (>30%), especially in case of travel-related infections (>50%), a macrolide antibiotic such as clarithromycin is first choice when antibiotic treatment is indicated (9).

Reactive arthritis and the Guillain-Barré syndrome (GBS) are post-infectious complications of *Campylobacter* enteritis. It is estimated that approximately 1-3% of *Campylobacter* infections is followed by an episode of arthritis (10). The duration of arthritis, which is often migratory, varies from several weeks or months up to one year and recovery is always complete. *Campylobacter-*

associated GBS occurs less frequently, estimated at 60 cases per year in The Netherlands, but is responsible for a considerable part of the health burden and costs due to *Campylobacter* infections. For the year 2000, the cost-of-illness due to *Campylobacter*-associated GBS in The Netherlands was estimated at € 3.4 million, whereas the cost-of-illness due to *Campylobacter* gastro-enteritis was estimated at € 15 million (10).

The Guillain–Barré syndrome

GBS is the most common cause of acute neuromuscular paralysis worldwide, with a global incidence rate of 1–2 cases per 100 000 people per year (11). GBS is an immune-mediated polyneuropathy characterized by an acute progressive, symmetrical motor weakness of the extremities and loss of tendon reflexes. Sensory and autonomous disturbances may also be present (12). The degree of paralysis varies widely and up to one-third of the patients need artificial respiration (13). Paralysis gradually resolves several weeks after the onset of symptoms, but recovery is not always complete.

Depending on the type of predominant nerve fibre damage, GBS can be divided into demyelinating and axonal forms. Acute inflammatory demyelinating polyneuropathy (AIDP) is the most frequent form of GBS in Northern America and Europe (14), whereas acute motor axonal neuropathy (AMAN) and acute motor-sensory axonal neuropathy (AMSAN) occur most often in China, Japan and Mexico (15-17). The Miller Fisher syndrome (MFS) is a rare variant of GBS, which is characterized by ophthalmoplegia, limb ataxia and areflexia in the absence of limb weakness (18).

Treatment of GBS patients primarily involves general supportive medical care. Plasma exchange and the intravenous administration of immunoglobulins have been shown to have a beneficial effect on the speed of recovery and residual functional deficits (19, 20). Despite adequate treatment, the mortality rate is approximately 5% and up to 20% of the patients suffers from disabling residual deficits (21).

In approximately two-third of GBS patients, an infectious illness is reported in the weeks before the onset of neurological symptoms (11, 21). *C. jejuni* has been identified as the most frequent triggering infectious agent, preceding GBS in 14–80% of all cases (22-26). However, it is estimated that only approximately 0.2-1 per 1,000 *Campylobacter* infections are followed by GBS (27-29). A preceding *C. jejuni* infection is associated with a severe, pure motor form of GBS and poor prognosis (30, 31). Other microorganisms significantly associated with the development of GBS are cytomegalovirus, Epstein–Barr virus, *Mycoplasma pneumoniae* and *Haemophilus influenzae* (13, 22).

The role of ganglioside mimicry in GBS

Although the pathogenesis of GBS has not yet been fully elucidated, studies have provided evidence that molecular mimicry plays an important role in triggering GBS (32). Molecular mimicry refers to a structural resemblance of microbial antigens with host structures (33). This resemblance induces a cross-reactive antibody and/or T cell response leading to the neurological symptoms. The lipo-oligosaccharide (LOS) in the cell wall of many *C. jejuni* strains has been shown to exhibit molecular mimicry with gangliosides in peripheral nerves (Figure1) (34). Gangliosides are membrane glycolipids that are highly enriched in nerve tissue. They are composed of a ceramide tail that is inserted into the cell membrane and a highly variable oligosaccharide part that contains one or more sialic acid molecules. In most GBS patients, high titres of anti-ganglioside antibodies are present in the acute phase serum (35, 36). Antibody reactivity against numerous gangliosides has been detected in GBS patients and the specificity ranges widely between patients. The specificity of the cross-reactive antibody response is associated with the clinical features of GBS. The presence of antibodies against GM1a, GD1a, GalNAc-GD1a and GM1b is associated with pure motor GBS, whereas anti-GQ1b reactivity is present in most patients with MFS and GBS with oculomotor symptoms and ataxia (25, 31, 37-40). This phenomenon may be explained by the relative abundance of a certain ganglioside in specific nerves or nerve structures (41, 42). The pattern of anti-ganglioside reactivity is also associated with the type of antecedent infection. Anti-GM1a and anti-GD1a reactivity have been

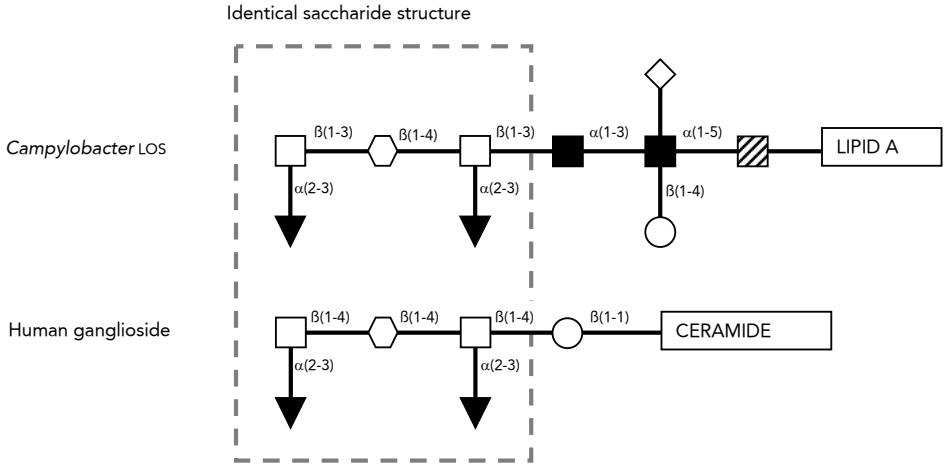

Figure 1. Molecular mimicry of *C. jejuni* LOS with the human ganglioside GD1a. The lipid A tail of the LOS is inserted into the cell membrane of the outer cell wall of *C. jejuni*. The ceramide tail of the ganglioside is inserted into the nerve cell membrane. The oligosaccharide chains of both LOS and ganglioside protrude from the outer parts of the *C. jejuni* cell wall and nerve cells, respectively.

□ galactose, ■ heptose, ▨ 3-deoxy-D-manno-octulosonic acid (KDO), ⬡ *N*-acetyl-galactosamine,
○ glucose, ▼ sialic acid, ▽ *N*-acetyl-D-mannosamine, ◇ (phosphoryl)ethanolamine

linked to a preceding *C. jejuni* infection, although this relationship was not found in all studies (14). Infection with CMV is associated with anti-GM2 reactivity and *M. pneumoniae* infection with antibodies against galactocerebroside (GalC) (43-45).

It has been demonstrated that anti-ganglioside antibodies of *C. jejuni*-infected GBS patients cross-react with *C. jejuni* LOS (46). Immunization of animals with *C. jejuni* LOS leads to a cross-reactive anti-ganglioside antibody response as well (47-50). Furthermore, it has been shown that the structure of the *C. jejuni* LOS corresponds with the specificity of the anti-ganglioside antibodies, both in GBS/MFS patients and in immunized animals (51, 52). However, *C. jejuni* strains isolated from patients with enteritis without GBS/MFS ("enteritis-only") can also harbour ganglioside-like structures in their LOS (53), indicating that ganglioside mimicry *per se* is not sufficient to induce GBS. Additional factors, both pathogen-, host- and/or environment-related, may also be necessary for the induction of a neuropathogenic immune response. It has been possible to induce an anti-ganglioside antibody response in various animal models after injection with *C. jejuni* LOS (47-50, 54). Yuki *et al.* described high anti-ganglioside antibody titers and acute flaccid paralysis in rabbits after immunization with purified GM1a ganglioside (55). More recently, the same group demonstrated an anti-GM1 IgG antibody response and flaccid limb weakness in rabbits after repeated sensitization with *C. jejuni* GM1-like LOS (56). There is only one report, which remains unconfirmed, however, of neuropathy development in chickens after a challenge with live *C. jejuni* bacteria (57).

C. jejuni strains associated with Guillain–Barré syndrome

Because GBS is a rare disease and stool cultures are often negative at the onset of neurological symptoms, the number of GBS-associated *C. jejuni* isolates available for study is limited. Several GBS/MFS-associated *C. jejuni* collections from different geographical areas have been extensively studied by various phenotyping and genotyping methods (58-60). So far, it has not been possible to identify a single factor common to all GBS-associated strains. However, in some parts of the world certain Penner HS-serotypes are over-represented among isolates from GBS cases. In GBS-associated *C. jejuni* strains from Japan, Mexico and the United States, a predominance of clonally related serotype HS:19 strains is observed (61-63). In South-Africa, most strains isolated from GBS patients are serotype HS:41, and are also clonally related (64, 65). In contrast, Dutch GBS/MFS-associated *C. jejuni* strains are genetically heterogeneous and the serotype distribution resembles that of the general *C. jejuni* population in The Netherlands (59). Comparison of a worldwide non-HS:19 collection of GBS-associated and enteritis-only strains performed by Engberg *et al.* also showed heterogeneity and did not reveal any GBS-specific markers (60).

Since the first report of a ganglioside mimic in the LOS of a GBS-associated *C. jejuni* strain in 1993 (66), a great variety of ganglioside-like structures have been identified in the LOS of GBS/MFS-associated strains. Mass spectrometry has revealed the presence of GM1a, GD3, GD1a and

GT1a mimics in GBS-associated strains and of GD3 mimics in MFS-associated strains (67-71). Serological studies have confirmed and extended these findings. However, as the specificity of serological assays varies, these are not suitable to determine the exact chemical structure of the LOS. Thus, serum reactivity for a specific ganglioside such as GM1a should be interpreted as the presence of a GM1a-like structure in the LOS, but not as a confirmation of the presence of the complete GM1a glycan portion. Only a few studies have compared the presence of ganglioside-like structures in the LOS between substantial collections of GBS/MFS-associated strains and enteritis-only strains. Ang *et al.* analyzed a group of Dutch non-clonal *C. jejuni* strains with several serological techniques and found that strains from GBS/MFS patients more frequently express ganglioside mimics than strains from uncomplicated enteritis patients (51). GM1a-like structures were detected in 85% of GBS-associated isolates and in 57% of enteritis-only strains, whereas GQ1b-like structures were expressed by 100% of MFS-associated strains and by only 9% of enteritis-only strains. Nachamkin *et al.* found that GBS-associated strains, both HS:19 and non-HS:19, were strongly associated with the expression of GD1a-like mimicry when compared to enteritis-only isolates (72).

The biosynthesis of ganglioside mimics in *C. jejuni*

LOS structure

Until several years ago, it was thought that the glycolipids in the *Campylobacter* cell wall, usually referred to as lipopolysaccharides (LPS), were composed of three consecutive regions: the lipid A part that is inserted into the cell wall, the core oligosaccharide and a long polysaccharide chain, called "high molecular weight lipopolysaccharide" (HMW LPS) (73). However, in 2000 it was demonstrated that the HMW LPS represents the capsule of *C. jejuni* which is not linked to the lipid A and core oligosaccharide (74).

Ganglioside mimics can be present in the core oligosaccharides; together with lipid A they are called the LOS (Figure 1). The inner core is relatively well conserved, usually consisting of 3-deoxy-*D*-manno-2-octulosonic acid (Kdo) and two heptose residues, with phosphoethanolamine or phosphate and glucose attached to the first heptose. The outer core structure is a highly variable oligosaccharide chain which often contains one or more sialic acid residues, an essential component of gangliosides.

LOS biosynthetic pathway

Many different enzymes are involved in the biosynthesis of the LOS outer core. The majority of these enzymes are glycosyltransferases, i.e. enzymes that attach a sugar molecule to another (sugar) molecule. Several other enzymes (synthetases) are involved in the biosynthesis of sialic acid.

The availability of the complete genome sequence of *C. jejuni* NCTC 11168 has allowed the identification of a cluster of genes involved in LOS biosynthesis (75). Since then, several studies

Table 1. *Campylobacter jejuni* LOS biosynthesis genes in class A, B and C LOS loci.

ORF# [a]	Cj# [b]	Gene [c]	LOS locus [d]	Proposed function
ORF1	Cj1133	*waaC*	A, B, C	Heptosyl transferase I
ORF2	Cj1134	*htrB*	A, B, C	Lipid A biosynthesis acyltransferase
ORF3	Cj1135		A, B, C	Two-domain glycosyltransferase
ORF4	Cj1136		A, B, C	ß-1,3-galactosyltransferase
ORF5		*cgtA*	A, B, C	ß-1,4-N-acetylgalactosaminyltransferase
ORF14	Cj1137c		C	Putative glycosyltransferase
ORF15	Cj1138		C	Putative glycosyltransferase
ORF6	Cj1139c	*wlaN, cgtB*	A, B, C	ß-1,3-galactosyltransferase
ORF7	Cj1140	*cst-II* (A/B) , *cst-III* (C)	A, B, C	α-2,3 or α-2,3/α-2,8-sialyltransferase
ORF8	Cj1141	*neuB*	A, B, C	Sialic acid synthetase
ORF9	Cj1142	*neuC*	A, B, C	N-acetylglucosamine-6P 2-epimerase
ORF10		neuA	A, B	CMP-sialic acid synthetase
ORF11			A, B	Putative acetyltransferase
ORF5/10	Cj1143	*neuA*	C	ß-1,4-N-acetylgalactosaminyltransferase, CMP-sialic acid synthetase
ORF16	Cj1144c		C	Hypothetical protein
ORF17	Cj1145c		C	Hypothetical protein
ORF12	Cj1146c	*waaV*	A, B, C	Putative glucosyltransferase
ORF13	Cj1148	*waaF*	A, B, C	Heptosyltransferase II

[a] the codes ORFx correspond to the gene numbering by Gilbert *et al.*(20).
[b] the codes Cjxxxx correspond to the gene numbering of strain NCTC11168.
[c] gene nomenclature as found in the literature.
[d] class of LOS locus in which the gene is present.

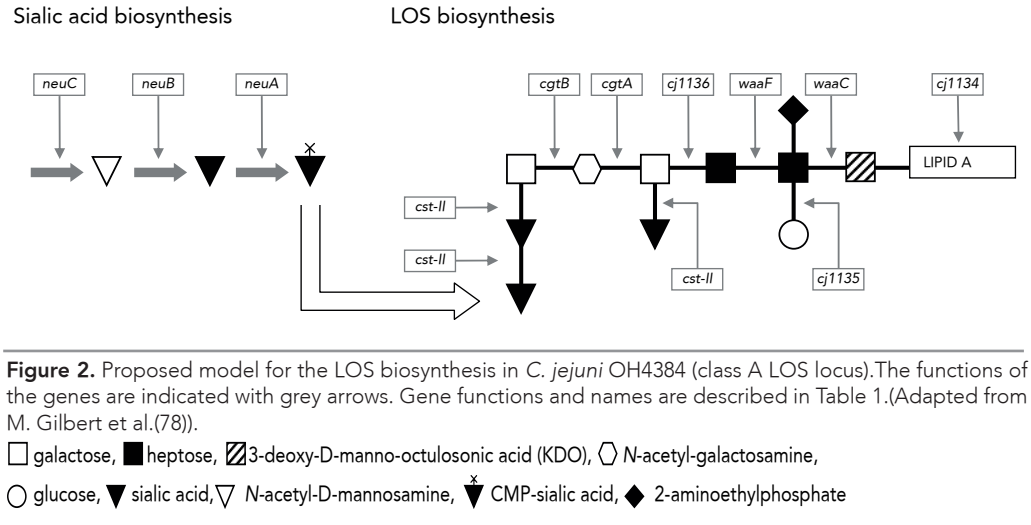

Figure 2. Proposed model for the LOS biosynthesis in *C. jejuni* OH4384 (class A LOS locus).The functions of the genes are indicated with grey arrows. Gene functions and names are described in Table 1.(Adapted from M. Gilbert et al.(78)).

have determined the function of specific LOS biosynthesis genes (Table 1). Based on these studies and on comparative data, Linton et al. proposed a model for LOS outer core biosynthesis in *C. jejuni* NCTC 11168 (76). Recently, Gilbert et al. proposed a biosynthetic pathway for the LOS core in *C. jejuni* OH4384, based on comparative genomics and *in vitro* expression assays of specific glycosyltransferases (Figure 2) (77).

Mechanisms for variation in LOS structure

The variability in LOS outer core structure is expected to be the result of underlying genetic mechanisms. Analysis of these mechanisms will provide a better understanding of the biosynthesis of ganglioside mimics and may help to further elucidate the pathogenesis of GBS. Comparative genomics studies of the LOS biosynthesis genes in various *C. jejuni* strains combined with *in vitro* enzymatic activity assays resulted in the identification of five different mechanisms that allow *C. jejuni* to vary the LOS outer core structure (Table 2) (78).

Table 2. Genetic mechanisms for variation in the LOS structure of *C. jejuni*

- Differences in gene content	
- Gene inactivation	- phase variation due to homopolymeric tracts
	- deletion or insertion of a single base (without phase variation)
	- single mutation
- Homologous enzymes with different acceptor specificities due to mutations	

1) *Different gene complement.* Initially, comparison of the LOS biosynthesis loci of 11 *C. jejuni* strains that express ganglioside mimics identified 3 different classes of the LOS locus (class A, B and C), based on variation in gene content. Additional analyses of strains without ganglioside mimics in their LOS outer core revealed multiple other classes (D to G) (Figure 3) and it is expected that new classes will be identified when more strains are studied (77, 79). There is extensive variation in the number of genes and in gene content. Some genes are unique for one class, whereas other genes occur in more than one class and 5 genes are present in all classes.

However, strains with the same LOS locus can express different LOS structures and even within one strain a mixture of LOS structures can be found. This is the result of other mechanisms that *C. jejuni* uses to turn on or off a gene or to change acceptor specificities of glycosyltransferases.

2) *Phase variation because of homopolymeric tracts.* Homopolymeric tracts are short sequence DNA repeats that can be involved in high frequency on-off switching of a gene. Slipped-strand mispairing that occurs during replication generates variation in tract length, which results in a mixture of in-frame and out-of-frame variants of a gene. This process is called phase variation. Homopolymeric tracts can be found in many LOS biosynthesis genes. Linton et al. demonstrated that variation in the poly-G tract of *orf6* resulted in the expression of either a GM1a or a GM2 ganglioside mimic in strain NCTC 11168 (80). Variation in the poly-G tract of *cgtA* was

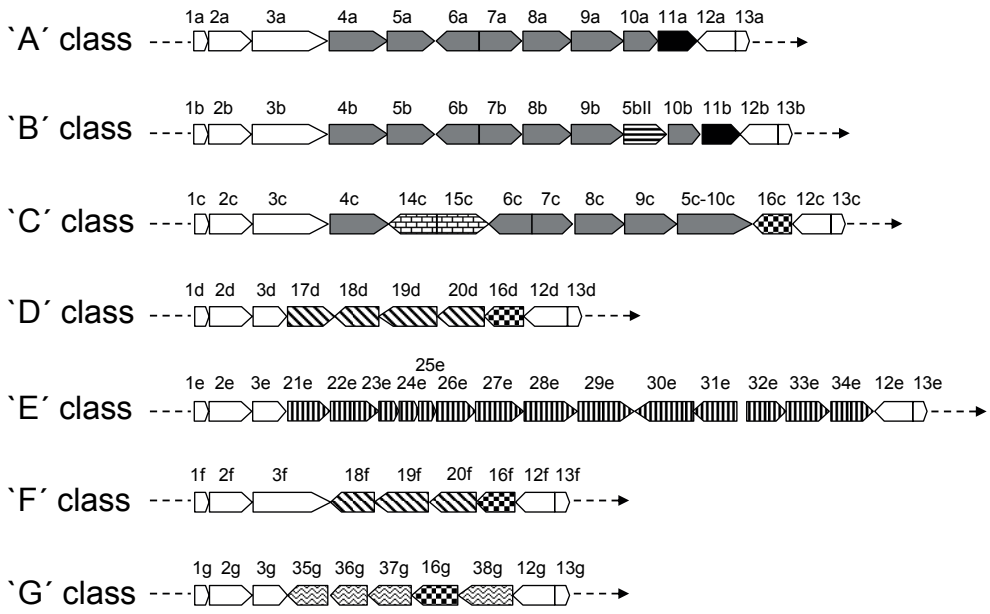

Figure 3. Genetic organization of the LOS biosynthesis loci of the different *C. jejuni* strains. The direction of the arrows indicates the direction of transcription. The filling of the arrows indicates in which LOS loci homologous genes are found (see legend below). Corresponding homologous genes have the same number with a letter for the LOS locus class added. Proposed functions for the class A, B and C genes are described in Table 1. (Adapted from M. Gilbert et al. (78)).

responsible for variants with GM2 and variants with GM3 mimicry in strain 81-176 (81).

3) *Gene inactivation by the deletion or insertion of a single base (without phase variation).* Genes can also be inactivated by frame-shift mutations that are not phase-variable, which means that there is no high-frequency on-off switching of the gene. For example, *cgtA* in OH4382 contains a stretch of 7 A bases instead of 8, which results in the expression of a truncated CgtA and a corresponding LOS outer core that is truncated after the inner galactose residue.

4) *Single mutation leading to the inactivation of a glycosyltransferase.* A single mutation that causes an amino acid change can result in an inactive glycosyltransferase. Such a mutation was responsible for the abolishment of the β-1,4-*N*-acetylgalactosaminyltransferase activity of the *orf5/10* fusion product, resulting in the expression of a truncated LOS in strain ATCC 43430 when compared with strain NCTC 11168.

5) *Single or multiple mutations leading to "allelic" glycosyltransferases with different acceptor specificities.* The acceptor specificities of the β-1,4-*N*-acetylgalactosaminyltransferase alleles (CgtA, CgtA-I, CgtA-II) from the classes A, B, and C were found to vary significantly. Some versions can only use a non-sialylated acceptor, others only a monosialylated acceptor and

others can use both mono- and disialylated acceptors. In most cases, the specificity corresponds with the natural acceptor: for example, if there is a single sialic acid on the inner galactose residue, the specificity of the corresponding CgtA is for a monosialylated acceptor.

Another example is provided by Cst-II, a sialic acid transferase. Some versions have α-2,3-sialyltransferase activity (monofunctional), whereas others have both α-2,3- and α-2,8-sialyltransferase activity (bifunctional). A bifunctional Cst-II is necessary for the expression of disialylated LOS (two sialic acid residues as a chain). Gilbert et al. demonstrated that one amino acid residue is essential for bifunctional activity, whereas two other amino acid residues were found to affect the relative ratios of α-2,3- and α-2,8-sialyltransferase activity (78).

Aims and outline of this thesis

The main aim of the research described in this thesis is to identify genetic markers for GBS in *C. jejuni*. Many studies have provided evidence for the hypothesis that molecular mimicry between *Campylobacter* LOS and gangliosides in human nerves plays a crucial role in the pathogenesis of GBS and MFS. Therefore, we first studied *Campylobacter* genes involved in LOS biosynthesis. We hypothesized that polymorphism in the LOS biosynthesis genes determines LOS structure and thereby the capability of a *C. jejuni* strain to induce a specific anti-ganglioside antibody response and clinical symptoms of GBS and MFS (Figure 4). Next, we aimed at the identification of potential genetic markers for GBS outside the LOS biosynthesis genes. Only a small proportion of infections with *C. jejuni* strains that express ganglioside mimics are followed by GBS or MFS. Therefore, it is likely that other pathogen- and/or host-related factors are also important for the development of neuropathy.

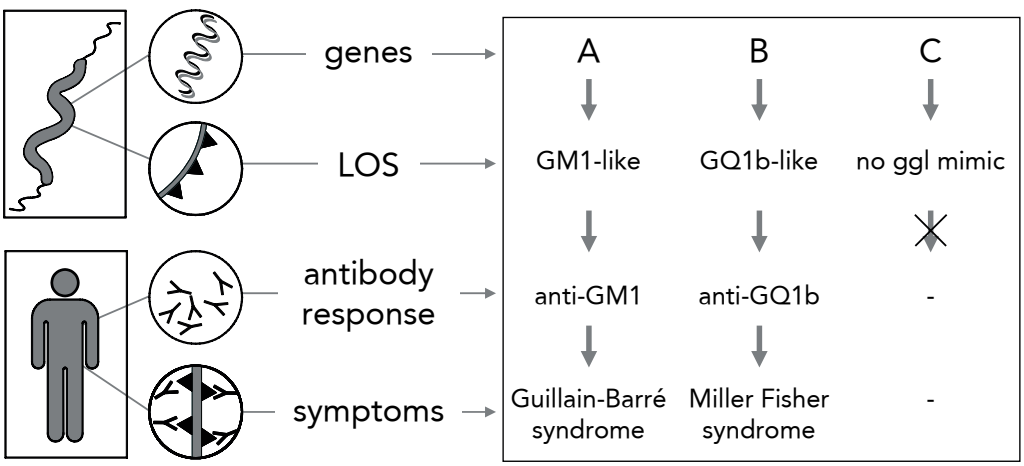

Figure 4. Model for *C. jejuni* LOS biosynthesis gene polymorphism as a determinant of LOS structure, anti-ganglioside antibody specificity and clinical symptoms.

In **Chapter 2**, we determined the types of LOS biosynthesis gene locus in our neuropathy-associated and control strains to identify potential GBS marker genes. We constructed gene knock-out mutants to investigate the role of these genes in ganglioside mimicry and induction of anti-ganglioside antibodies. In **Chapter 3**, we analyzed the individual LOS biosynthesis genes with PCR-RFLP to investigate the presence of additional markers for GBS. **Chapter 4** describes how a *C. jejuni* strain may have acquired a GBS-related LOS biosynthesis locus. In **Chapter 5** we investigated whether co-infections with multiple *C. jejuni*-strains occur in GBS patients and we further characterized these strains. In **Chapter 6** we assessed the relationship between genetic polymorphism in LOS genes, the exact LOS structures as determined by mass-spectrometry techniques and clinical symptoms in the patients. Knowledge of the exact LOS structures enabled us to investigate the origin of antibodies against ganglioside complexes (**Chapter 7**). Finally, we used high-throughput AFLP analysis, a genome-wide genotyping technique, to investigate the presence of GBS markers located outside the LOS biosynthesis gene locus (**Chapter 8**).

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CHAPTER 2

The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in the Guillain-Barré syndrome

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Abstract

Molecular mimicry of *Campylobacter jejuni* lipo-oligosaccharides (LOS) with gangliosides in nervous tissue is considered to induce cross-reactive antibodies leading to the Guillain-Barré syndrome (GBS), an acute polyneuropathy. To determine whether specific bacterial genes are crucial for the biosynthesis of ganglioside-like structures and the induction of anti-ganglioside antibodies, we characterized the *C. jejuni* LOS biosynthesis gene locus in GBS-associated and control strains. We demonstrated that specific types of the LOS biosynthesis gene locus are associated with GBS and with the expression of ganglioside mimicking structures. *Campylobacter* knockout mutants of two potential GBS marker genes, both involved in LOS sialylation, expressed truncated LOS structures without sialic acid, showed reduced reactivity with GBS patient serum and failed to induce an anti-ganglioside antibody response in mice. To our knowledge, we demonstrate for the first time that specific bacterial genes are crucial for the induction of anti-ganglioside antibodies.

Introduction

Autoimmune diseases are often preceded by an infectious illness. Molecular mimicry between microbial antigens and structures in host tissue has been implicated as a mechanism for triggering a cross-reactive immune response after an infection (1). There is strong but indirect evidence for the pathogenic role of molecular mimicry in the Guillain-Barré syndrome (GBS), an acute peripheral polyneuropathy and the most frequent cause of acute neuromuscular paralysis (2). Therefore, GBS is an excellent model disease to study both microbial and host factors involved in molecular mimicry.

The most frequently identified triggering agent of GBS is *Campylobacter jejuni*, a spiral-shaped gram-negative bacterium (3, 4). *C. jejuni* is the leading causative agent of bacterial gastroenteritis worldwide and it has recently also been associated with neoplastic disease of the gut (5). Acute phase sera of GBS patients contain high titers of antibodies directed against gangliosides, membrane glycolipids that are highly enriched in nervous tissue (6). Biochemical and serological studies have identified various ganglioside-mimicking structures in the lipo-oligosaccharide (LOS) of the *Campylobacter* cell wall (7) and cross-reactive antibodies between *Campylobacter* LOS and gangliosides have been demonstrated in serum from GBS patients (6). Ganglioside-mimicking structures are more frequently found in neuropathy-associated *C. jejuni* strains compared to strains isolated from patients with diarrhea (8). An important feature in ganglioside mimicry is the presence of sialic acid (*N*-acetylneuraminic acid) in both LOS and gangliosides (9).

Recently, we described a collection of geographically clustered but genetically heterogeneous *C. jejuni* strains isolated from Dutch patients with GBS or its variant the Miller Fisher syndrome (MFS) (10, 11). Characterization of the isolates by phenotypic and molecular methods showed that no clustering of GBS/MFS-associated strains occurred when these were compared to control strains (10, 12).

The availability of a database with detailed serological and clinical data of the Dutch GBS/MFS patients provides a unique opportunity for a systematic search for bacterial GBS/MFS-associated virulence factors and correlations with specific immune responses and clinical presentation. We recently reported the association between the presence of the *campylobacter sialic acid transferase-II* (*cst-II*) gene and the expression of a GQ1b-like structure in the bacterial LOS (13). Based on these findings, we hypothesized that the presence of certain *C. jejuni* genes involved in LOS biosynthesis may be crucial for the induction of the anti-ganglioside immune response leading to GBS. Therefore, we analyzed the LOS biosynthesis gene locus of GBS/MFS-associated *C. jejuni* strains. We found that specific types of the LOS biosynthesis locus are associated with GBS and this finding led to the identification of potential GBS marker genes in *C. jejuni*. Functional analysis of *Campylobacter* gene knockout mutants, including mice immunization experiments, demonstrated that these genes are crucial for the induction of anti-ganglioside antibodies.

Results

Specific classes of the LOS biosynthesis gene locus are associated with neuropathy and ganglioside mimicry

In *C. jejuni*, the genes involved in LOS biosynthesis are clustered in the LOS biosynthesis gene locus ("LOS locus") (14). Variation in gene content of the LOS locus is an important mechanism for variation in LOS structure between different *C. jejuni* strains. Previously, we had described three different gene compositions or "classes" of the LOS biosynthesis gene locus in *C. jejuni* (15). Since then, the DNA sequences of several additional *C. jejuni* LOS loci were deposited in GenBank™ (http://www.ncbi.nlm.nih.gov/Genbank/) and there are now five distinct classes (Figure 1).

To study whether certain classes are more prevalent among neuropathy-associated *C. jejuni* strains, we determined the class of LOS locus (class A to E) in a collection of 21 neuropathy-

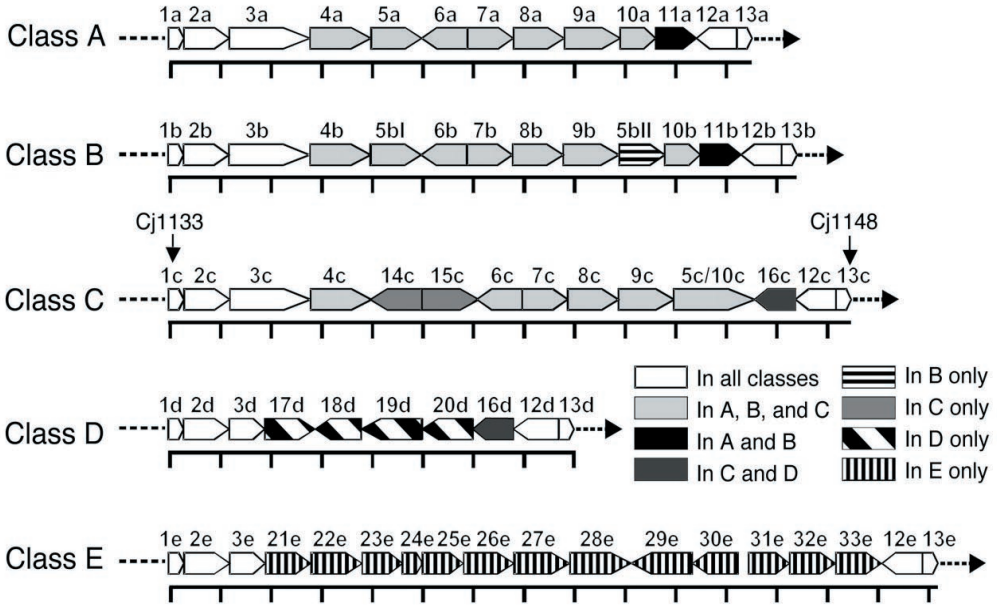

Figure 1. Genetic organization of the five different classes of the *C. jejuni* LOS biosynthesis locus. The distance between the scale marks is 1 kb. The direction of the arrows indicates the direction of transcription. Corresponding homologous genes have the same number with a letter for the LOS locus class added. For *orf1c* and *orf13c* the corresponding "Cj" gene numbers of the genome strain NCTC 11168 are given. The five LOS classes are based on DNA sequences of the following strains (Genbank™ accession number). Class A: OH4384 (AF130984), OH4382 (AF167345), HS:4 (AF215659), HS:10 (AF400048), HS:19 (AF167344), HS:41 (AY044868); class B: HS:23 (AF401529), HS:36 (AF401528); class C: NCTC 11168 (AL139077), HS:1 (AY044156), HS:2 (AF400047); class D: LIO87 (AF400669) and class E: 81116 (AF343914 and AJ131360). The proposed functions for the *orfs* are described in Supplementary Table 1.

associated and 21 control *C. jejuni* strains isolated from patients with uncomplicated enteritis. All the strains used in this study were positive for one of the five identified LOS locus classes. In addition, we analyzed the individual class A/B and C genes by PCR RFLP and hybridization analysis and found that the LOS gene content in the strains was in agreement with their class of LOS locus (data not shown). The class A LOS locus was overrepresented in the GBS-associated strains compared to the control strains (53% versus 14%, $P = 0.02$; Table 1). In contrast, all four MFS-associated strains contained a class B locus, which was detected in only 33% of control strains ($P = 0.03$; Table 1) and 18% of GBS-associated strains ($P < 0.01$).

Studying the expression of ganglioside-like structures in relation to the class of LOS locus, we found that GM1-like structures were associated with a class A locus ($P < 0.01$), whereas GQ1b-like structures were predominantly expressed by strains with a class B locus ($P < 0.01$; Table 2). Although the class A locus was associated with both GBS and the presence of a GM1-like structure, a GM1-like structure was not detected more frequently in GBS-associated strains (13/17 GBS strains vs. 12/21 control strains, $P = 0.3$). In 8/11 strains with a class D or E locus ganglioside-like structures were not detected, which is in accordance with the absence of genes involved in the biosynthesis or transfer of sialic acid in these classes (Table 2, Figure 1). These

Table 1. LOS biosynthesis loci in *C. jejuni* strains from patients with GBS, MFS and uncomplicated enteritis

	No. of strains associated with:			
LOS locus class	GBS (n=17)	MFS (n=4)	enteritis (n=21)	P
A (n=12)	9	0	3	0.02[a]
B (n=14)	3	4	7	0.03[b]
C (n=5)	2	0	3	NS
D (n=4)	2	0	2	NS
E (n=7)	1	0	6	NS

[a]GBS vs enteritis
[b]MFS vs. enteritis

results indicate that genes that are unique to the class A and B loci and genes that are involved in sialic acid biosynthesis or transfer may be crucial for the induction of neuropathogenic cross-reactive antibodies and may thus be considered as GBS marker genes.

Mutagenesis of putative GBS marker genes

To study whether potential GBS marker genes are indeed crucial for the induction of potentially neuropathogenic cross-reactive antibodies, we constructed *Campylobacter* knockout mutants of three potential GBS marker genes. *Open reading frame (orf) 11* is unique for the class A and B loci (Figure 1). Its function is unknown, but sequence homology with an enzyme of *Rhodobacter capsulatus* suggests that *orf11* encodes an acetyltransferase (16). The *cst-II*

Table 2. LOS biosynthesis loci and presence of ganglioside-like structures in *C. jejuni* strains

LOS locus class	No. of strains with a ganglioside-like structure in the LOS[a]				P
	GM1 (n=27)	GQ1b (n=9)	other (n=30)	none (n=8)	
A (n=12)	12	1	10	0	<0.01[b]
B (n=14)	9	8	14	0	<0.01[c]
C (n=5)	5	0	3	0	NS
D (n=4)	0	0	1	3	0.01[d]
E (n=7)	1	0	2	5	<0.01[d]

[a] a strain can express more than one ganglioside-like structure in its LOS. Therefore, the sum of strains in a row (GM1 + GQ1b + other + none) is higher than the actual number of strains with the corresponding LOS locus type.
[b] GM1 vs. non-GM1
[C] GQ1b vs. non-GQ1b
[d] none vs. any

gene (*orf7*) encodes an enzyme (Cst-II) that is involved in the transfer of sialic acid to the LOS backbone. *Orf10* encodes a CMP-sialic acid synthetase and is involved in the biosynthesis of the sialic acid donor used by Cst-II.

Cst-II and *orf11* single mutants and an *orf10/orf11* double mutant were produced in two class A GM1-expressing GBS-associated *C. jejuni* strains (GB2 and GB11) by insertion of a chloramphenicol resistance (Cm^r) cassette (Figure 2). The cassette was inserted with the same transcriptional polarity as that of the mutated genes. For all mutants, possible double crossover events were verified by PCR. Southern blot analysis confirmed single integration of the Cm^r cassette into the correct position on the chromosome (data not shown).

LOS structure analysis of wild types and mutants

To determine the effect of the gene inactivations on the LOS structure, we performed mass spectrometry analysis and identified a mixture of GM1- and GD1a-like structures for both GB2 and GB11 wild type LOS (Figure 3). The sets of triply-charged ions corresponding to GM1- and GD1a-like structures were of similar intensities (data not shown), which suggests that these two structures are present in approximately equimolar amounts, assuming that the ionization efficiency is proportional to the amount of a species in the sample. Both the *cst-II* and *orf10/11* mutants of GB2 and GB11 expressed a mixture of three non-sialylated structures. The main fraction consisted of asialo-GM3, but asialo-GM2 and asialo-GM1 structures were also present (Figure 3). No structural differences were observed between the wild types and the *orf11* mutants. This indicates that the truncated LOS structures as observed in the *orf10/orf11* mutants were caused by the inactivation of *orf10* and not *orf11*. Detailed results of the mass spectrometry analysis can be found in Supplemental Table 2.

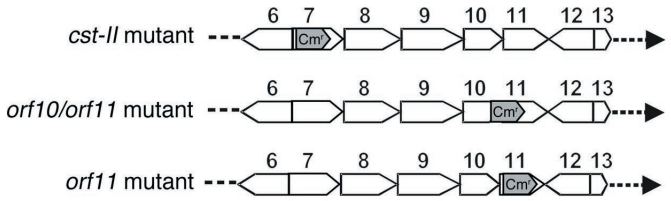

Figure 2. Schematic representation of the mutants used in this study. *Orf6* to *orf13* of the class A LOS locus are displayed. The positions of the 0.7-kb chloramphenicol resistance (CmR) cassette are indicated with grey arrows. Both *orf10* and *orf11* are inactivated in the *orf10/orf11* mutant.

Strain	Structure	Ganglioside mimic
wild type GB2/GB11	Gal-GalNAc-Gal-Hep-Hep- | | NeuAc Glc	GM1
	Gal-GalNAc-Gal-Hep-Hep- | | | NeuAc NeuAc Glc	GD1a
cst-II and *orf10/orf11* mutants GB2/GB11	Gal-Hep-Hep- | Glc	no
	GalNAc-Gal-Hep-Hep- | Glc	no
	Gal-GalNAc-Gal-Hep-Hep- | Glc	no
orf11 mutants GB2/GB11	like wild types	like wild types
NCTC 11168	Gal-GalNAc-Gal-Gal-Hep-Hep- | | | | NeuAc Gal Glc Glc	GM1
	GalNAc-Gal-Gal-Hep-Hep- | | | | NeuAc Gal Glc Glc	GM2

Figure 3. Molecular mimicry between gangliosides and LOS outer cores of wild type and mutant *C. jejuni* strains. LOS outer core structures of GB2 and GB11 wild type and mutant strains were determined by mass spectrometry analysis. The LOS outer core structure of the "genome" strain NCTC 11168 was described by St. Michael et al. (35). Note that all strains express a mixture of different LOS structures. The *cst-II* and *orf10/orf11* mutants of GB2 and GB11 did not express ganglioside mimics but a mixture of three non-sialylated structures. No structural differences were observed between the wild types and the *orf11* mutants.

Sialic acid mutants have a reduced reactivity with patient serum

Analysis of the LOS by gel electrophoresis revealed faster migrating LOS cores for the GB11 *cst-II* and *orf10/orf11* mutants compared to the wild type, confirming that these mutants have a truncated LOS (Figure 4a). The *orf11* mutant LOS showed migration patterns identical to wild type LOS. Results for the GB2 mutant were identical to those obtained with GB11 (results not shown). These findings are concordant with the structures found by mass spectrometry.

To determine whether the gene inactivations influenced the reactivity of the LOS with the antibodies in the serum from GBS patients, we performed an immunoblot analysis with the serum of the GB11 patient and wild type and mutant purified LOS fractions. Previous studies showed that this serum contains high levels of anti-GM1 antibodies (8). Reactivity of GB11 patient serum was reduced for the *cst-II* and *orf10/orf11* mutants (Figure 4b) but remained the same for the *orf11* mutant when compared to the wild types. For the *cst-II* and *orf10/orf11* mutants, the reactivity with cholera toxin, a ligand for GM1-oligosaccharide structures, was almost completely lost (Figure 4c). These results indicate that genes involved in sialic acid biosynthesis and transfer are important for the expression of ganglioside-like LOS structures that are capable of inducing potentially pathogenic autoantibody responses. Although strain GB11 and the "genome" strain NCTC 11168 both express GM1 mimics in their LOS (which was confirmed by their reactivity with cholera toxin, Figure 4c), the GB11 patient serum did not react with the LOS of NCTC 11168 (Figure 4b), which has been described previously (17). This observation illustrates the fine specificity of the antibody response in patient GB11, as the GB11 and NCTC 11168 strains have different LOS structures with a common GM1 mimic (Figure 3).

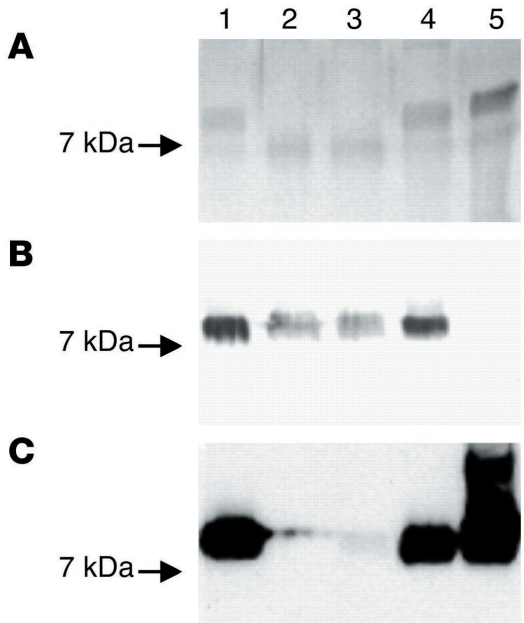

Figure 4. SDS PAGE analysis of LOS from GB11 wild type and mutants. The genome strain NCTC 11168 was included as a control. Lane 1, wild type GB11; lane 2, GB11 *cst-II* mutant, lane 3, GB11 *orf10/orf11* mutant, lane 4, GB11 *orf11* mutant, lane 5, NCTC 11168. (A) Silver staining of the LOS revealed faster migrating LOS cores for the GB11 *cst-II* and *orf10/orf11* mutants compared to the wild type, indicating that these mutants have a truncated LOS (Figure 4a). The *orf11* mutant LOS showed migration patterns identical to wild type LOS. (B) A Western blot incubated with GB11 patient serum showed a reduced reactivity for the *cst-II* and *orf10/orf11* mutants but unchanged reactivity for the *orf11* mutant when compared to the wild types. (C) For the *cst-II* and *orf10/orf11* mutants, the reactivity with cholera toxin, a ligand for GM1-oligosaccharide structures, was almost completely lost. Reactivity with the *orf11* mutant remained unchanged.

Sialic acid mutants fail to induce anti-ganglioside antibody responses in mice

To demonstrate that the truncated LOS structures in the mutants do not induce autoantibodies, we immunized GD2/GM2 synthase knockout mice. These mice lacking complex gangliosides are immune-naive hosts that can be used to raise high-titer anti-ganglioside antibody responses (18). Immunization with wild type GB11 lyophilized whole bacteria resulted in high IgG serum responses against GD1a, a ganglioside containing two sialic acid residues, in two of two mice (Figure 5a). We could not detect antibody reactivity against GM1 (results not shown). None of the mice injected with the *cst-II* and *orf10/orf11* mutants produced anti-GD1a antibodies (Figure 5b,c). However, all six mice produced anti-asialo-GM1 antibodies, as can be expected from the truncated LOS structures in the *cst-II* and *orf10/orf11* mutants (Figure 5f,g). Two of three mice immunized with the *orf11* mutant strain produced GD1a antibodies (Figure 5d). Low asialo-GM1 titers were also found in three of three mice immunized with the *orf11* mutant (Figure 5h). These results indicate that genes involved in sialylation of the LOS are crucial for the induction of cross-reactive anti-ganglioside antibodies.

Discussion

We have shown that specific types of the *C. jejuni* LOS biosynthesis gene locus are clearly associated with immune-mediated neuropathy and with the presence of ganglioside-mimicking structures in the LOS. Moreover, using *Campylobacter* gene knockout mutants, we demonstrate here for the first time that genes involved in sialylation of the LOS are crucial for the induction of anti-ganglioside antibodies.

Diversity in the content of genes involved in LOS biosynthesis results in the variety of LOS structures that is observed in *C. jejuni*. In this study, we identified two new classes of the LOS biosynthesis gene locus, in addition to three previously described classes (15, 19). At the time of writing, DNA sequences of additional *C. jejuni* strains are accessible through GenBank™ and new, not earlier reported LOS gene combinations can be identified. However, all our strains had a positive PCR test for one of the five LOS locus classes described in this study, indicating that our tests completely covered the spectrum of different LOS loci present in this collection of *Campylobacter* strains.

The expression of ganglioside-mimicking structures in *Campylobacter* LOS is considered to be essential for the induction of autoantibodies leading to GBS. Indeed, we previously reported that ganglioside-mimicking structures are more frequently present in neuropathy-associated strains compared to strains isolated from patients with uncomplicated enteritis (8). In contrast, most of the *Campylobacter* strains whose LOS structure has been chemically characterized and reported in the literature express ganglioside-mimicking structures. The number of strains that have been analyzed, however, is small and may not be representative. Our study demonstrates that *C. jejuni* needs specific gene combinations to express ganglioside mimics. Only three of the five identified classes of the LOS locus, i.e. classes A, B and C, contain genes that are involved in

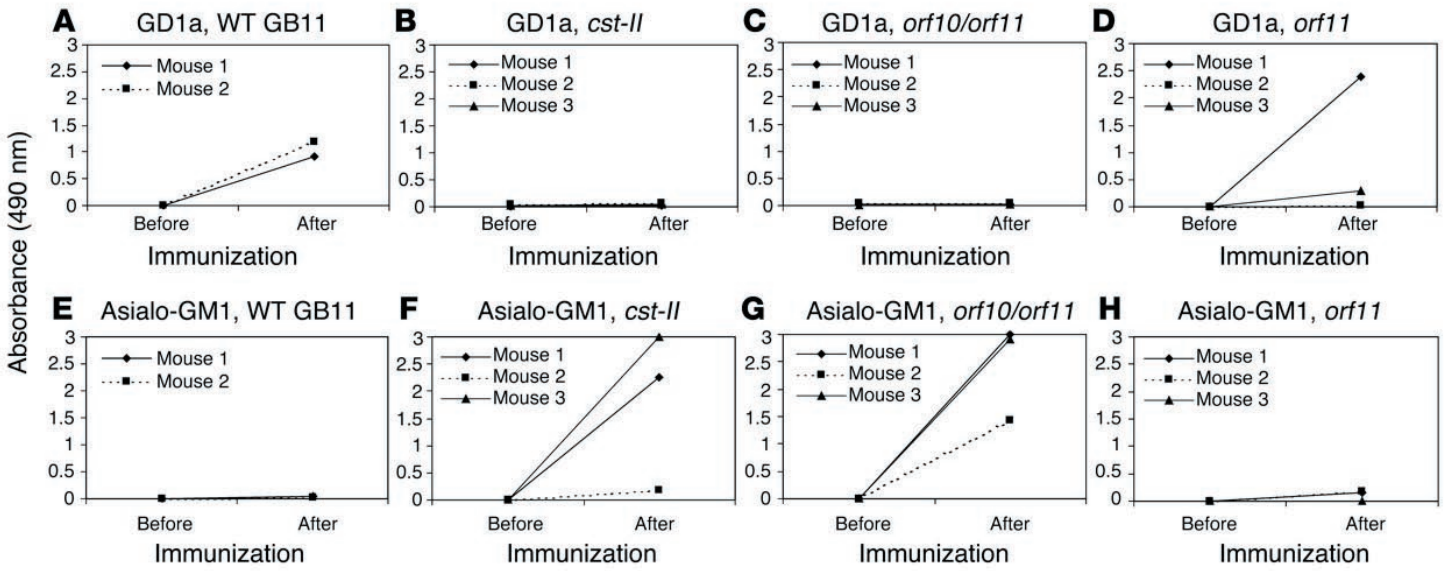

Figure 5. Anti-glycolipid antibodies in serum of mice before and after immunization with wild type and mutant GB11 *C. jejuni* strains. (A, D) When sialic acid was present on the LOS core (wild type and *orf11* mutant), anti-GD1a antibodies were induced in mice. (B, C) Anti-GD1a antibody responses were not detected in mice immunized with non-sialylated LOS (*cst-II* and *orf10/orf11* mutants). (F, G) Non-sialylated LOS (*cst-II* and *orf10/orf11* mutants) induced high asialo-GM1 antibody responses. (E, H) Asialo-GM1 antibody responses were absent or low after immunization with the *orf11* mutant and wild type.

the biosynthesis and transfer of sialic acid, an essential component of gangliosides. We showed that these LOS loci are present in the large majority of strains expressing ganglioside mimics, including the neuropathy-associated strains, whereas class D and E loci are more frequently found in strains lacking ganglioside mimics. In three class D/E strains, ganglioside-like structures other than GM1 or GQ1b were detected (Table 2). The sera used in the serological assay for the detection of these "other" ganglioside-like structures may also react with asialo-GM1-like structures lacking sialic acid. Therefore, the detection of an "other" ganglioside-like structure does not necessarily imply that the strain is able to synthesize and transfer sialic acid. However, we cannot exclude the possibility that some strains could have both class D or E genes and sialic acid biosynthesis genes that have diverged from their homologues in classes A/B/C.

Interestingly, we found that the class A locus is associated specifically with GBS and the presence of a GM1-like structure, whereas the class B locus is associated with MFS and the presence of a GQ1b-like structure. The presence of anti-GM1 antibodies has been shown to be associated with a preceding *Campylobacter* infection (3). We found that GM1-like structures can be expressed by strains with various LOS locus classes. However, the association of a class A LOS locus with both GBS and the presence of a GM1-like structure suggests that class A strains expressing GM1-like LOS structures are more likely to induce GBS compared to non-A strains with a GM1-like LOS.

The Miller Fisher sydrome, a variant of GBS, has consistently been associated with preceding *C. jejuni* infection. Several authors have demonstrated that GQ1b-like structures occur more frequently in MFS-associated strains and are associated with anti-GQ1b antibody reactivity and the presence of oculomotor symptoms in patients with GBS or MFS (8, 20). Although GQ1b-like structures were detected in both class A and class B strains, we found that only the class B locus was significantly associated with presence of a GQ1b-like structure and that all our MFS-associated strains contained a class B locus. However, not all class B strains expressed GQ1b-like structures. This may be explained by mechanisms such as phase variation or point mutations that lead to gene inactivations resulting in the absence of expression of GQ1b-like structures in some class B strains (21, 22). In conclusion, we have now identified specific molecular substrates in *C. jejuni* involved in the induction of GBS or MFS.

To our knowledge, this is the first study that analyzed the LOS locus of a large collection of neuropathy-associated and control *C. jejuni* strains. Previously, it was demonstrated that two closely related GBS associated strains contained a class A locus (16). Very recently, we demonstrated that the strain GB11 that we used in this study has probably acquired its class A locus through horizontal gene transfer (17). Another recent report did not find an association between GBS and LOS biosynthesis genes using a whole genome DNA microarray (23). This microarray, however, only contained LOS genes present in a class C locus and would therefore not be able to detect the association described here.

Based on the results of the LOS locus analysis, we hypothesized that genes unique for the class A and B loci or genes that are involved in sialic acid biosynthesis and transfer are crucial for the induction of neuropathogenic cross-reactive antibodies. To test this hypothesis, we

constructed a *Campylobacter* knockout mutant of *orf11*, encoding a putative acetyltransferase with unknown function in LOS biosynthesis and the only gene that is unique to class A and B loci. Several genes are involved in the sialylation of *Campylobacter* LOS (Supplemental Table 1). Inactivation of either of these genes will probably lead to identical changes in LOS structure, i.e. truncated LOS lacking sialic acid. We constructed knockout mutants of two such genes: *orf10*, a CMP-sialic acid synthetase, and *cst-II*. *Cst-II*, present in class A and B loci, encodes a bifunctional sialic acid transferase which has been characterized in detail and which has been associated with the presence of a GQ1b-like structure (13, 15, 24). *Cst-II* has a sialyltransferase homologue in the class C locus, called *cst-III* (53% homology), but the bifunctional (α-2,3/α-2,8) sialyltransferase activity that is probably necessary for the biosynthesis of GQ1b-like structures has so far only been detected for *cst-II* (15). *Orf10* was inactivated along with the adjoining *orf11* in a so-called double mutant (Figure 2). Because the *orf11* single mutants did not show any differences compared to the wild types, we assumed that the differences observed in the *orf10/orf11* double mutant were the result of the inactivation of *orf10*. Mutants were constructed of two class A GBS-associated strains that expressed GM1-like structures in their LOS. We were unsuccesfull in our efforts to construct mutants of MFS-associated strains expressing GQ1b-like structures as unfortunately these strains did not appear to be transformable (results not shown).

Mass spectrometry analysis revealed a mixture of two different sialylated ganglioside-mimicking structures in the wild-type GB2 and GB11 strains. Inactivation of *cst-II* and *orf10* resulted in a mixture of three truncated LOS structures lacking sialic acid. Two of these structures not only lacked sialic acid when compared to the wild type, but also the outer Gal or Gal-GalNAc residues of the LOS backbone (Figure 3). This observation is consistent with the lower activity of the β-1,4-*N*-acetylgalactosaminyltransferase (CgtA) on non-sialylated acceptors that was previously reported for CgtA from *C. jejuni* OH4384 (16). Inactivation of *orf11* did not result in LOS structure differences with the wild type strains. Therefore, the function, if any, of this gene in LOS biosynthesis and the pathogenesis of GBS remains unknown. In summary, mass spectrometry analysis revealed that genes involved in sialylation are necessary for the biosynthesis of ganglioside mimics in *Campylobacter* LOS. Other studies have demonstrated the role of *cst-III* (*orf7c*), *neuB1* (*orf8c*) and *neuC1* (*orf9b* and *orf9c*) in the synthesis of ganglioside mimics (21, 25, 26).

Inactivation of *cst-II* and *orf10* resulted in reduced reactivity of GB11 patient serum with mutant LOS, providing support for their importance in the induction of autoantibodies in GBS. Although residual reactivity was seen, this was probably caused by anti-asialo-GM1 antibodies or anti-GM1 and anti-GD1b antibodies that crossreact with asialo-GM1 structures in the mutant LOS. It has previously been demonstrated that the GB11 patient serum contains antibodies directed against GM1 (high titer), asialo-GM1 (low titer) and GD1b (high titer), all containing the terminal disaccharide Gal-GalNAc (8). Although non-sialylated glycoconjugates are present on nerve tissue, so far anti-asialo-GM1 antibodies have not been associated with GBS. The complete absence of reactivity of cholera toxin with *cst-II* and *orf10/orf11* mutant LOS further confirms the crucial role of *cst-II* and *orf10* in the biosynthesis of ganglioside mimics.

The mice immunization experiments conducted in this study provide additional evidence for the necessity of sialylation genes for the induction of autoantibodies. Both *cst-II* and *orf10/orf11* mutants failed to induce anti-ganglioside antibodies in mice, in contrast to the wild type strains. Mice injected with the *cst-II* and *orf10/orf11* mutants produced serum antibodies against the non-sialylated glycoconjugate asialo-GM1, but these antibodies have not yet been associated with GBS. Anti-GD1a but no anti-GM1 antibodies were detected in mice immunized with the GB11 wild type strain. This is in contrast with earlier findings that GB11 also expresses a GM1-like LOS structure and that GB11 patient serum contains high levels of anti-GM1 antibodies. However, it has been observed previously that immunizaton with both GD1a and GM1 induced primarily anti-GD1a responses in mice, whereas rabbits only had anti-GM1 antibodies (27, 28). Thus, anti-ganglioside antibody responses may be species dependent.

The associations that we found are not absolute. As host factors also play an important role in the pathogenesis of GBS, infection with a *C. jejuni* strain that expresses ganglioside-like structures is not sufficient to trigger GBS. In addition, a variety of ganglioside mimics, produced by different LOS classes, have been associated with GBS with diverse clinical manifestations (8, 20, 29). Furthermore, it is not clear at present whether all bacterial factors involved in the development of GBS/MFS have been identified.

After the demonstration of anti-ganglioside antibodies in GBS/MFS patient sera, the subsequent identification of ganglioside mimics in *Campylobacter* LOS and the specific host responses towards these epitopes, we now also demonstrate that the microbial gene repertoire is an important factor in the initiation of post-infectious autoimmune disease. The markers described in the current communication may facilitate the search for mechanisms of microbial pathogenicity and be helpful in the development of new molecular diagnostic tools for identifying *C. jejuni* strains with an increased ability to induce GBS/MFS. Furthermore, increased insight in the biosynthesis of ganglioside-mimicking structures may ultimately lead to the development of new treatment strategies and interventions (30).

Methods

C. jejuni strains

Seventeen GBS-associated and four MFS-associated *C. jejuni* strains used in this study were isolated from GBS/MFS patients from The Netherlands and Belgium between 1991 and 1999 (10). GB13 and GB26 were cultured from the diarrheal stools of family members of two GBS patients (31). Twenty-one control *C. jejuni* strains were isolated from sporadic Dutch enteritis patients without neurological symptoms between 1990 and 1999 (8). Ten reference *C. jejuni* Penner serotypes (HS:1, HS:2, HS:3, HS:4, HS:10, HS:19, HS:23, HS:35, HS:36 and HS:64) and the "genome" strain NCTC 11168 (19) were also included as controls for the PCR tests. Characterization of the isolates by phenotypic and molecular methods showed that no clustering of GBS/MFS-associated strains occurred when these were compared to control strains (10, 12).

Growth conditions and DNA isolation

For PCR analysis, *C. jejuni* strains were cultured for 24-48 hours on blood agar plates in a micro-aerobic atmosphere at 37°C. DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). For other experiments, *C. jejuni* wild type strains were grown at 37°C on Mueller-Hinton agar plates supplemented with Modified Preston Campylobacter Selective Supplement (MPCSS, Code SR0204E, Oxoid) in a micro-aerobic atmosphere. The mutant strains were grown on Mueller-Hinton agar plates supplemented with MPCSS and chloramphenicol, at a concentration of 20 µg/ml. For cloning, the *E. coli* strain JM109 (Promega) was grown on 2YT agar with 40 µg/ml ampicillin and 20 µg/ml chloramphenicol.

Serological methods

The ganglioside mimics in the LOS of the various strains were determined using polyclonal and monoclonal anti-ganglioside antibodies as described previously (8). An inventory was made for GM1-like, GQ1b-like and, collectively, other ganglioside-like structures.

Determination of the LOS locus class

To determine the class of LOS locus in *C. jejuni* strains, we developed specific primersets for the classes A/B, C, D and E, based on the DNA sequence of a gene unique for the LOS locus class(es) involved (Supplemental Table 1). To discern between classes A and B, we developed a primerset that was based on the DNA sequence of *orf5-II*. PCR assays were performed using a Biomed Thermal Cycler (Model 60) with a program consisting of 40 cycles of 1 min 94°C, 1 min 52°C, 2 min 74°C. Per reaction approximately 50 ng of template DNA was used in a buffer system consisting of 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton-X100, 0.2 mM of each of the deoxyribonucleotide triphosphates (Promega) and 0.2 units SuperTaq polymerase (HT Biotechnology). Timing needed to be adjusted for some of the amplifications. The selective PCR tests were validated with a panel of *C. jejuni* strains with a known LOS locus class.

Cloning and mutagenesis

The target genes (*cst-II*, *orf10/orf11* and *orf11*) and approximately 700 bp of flanking sequence were amplified and cloned into the pGem-Teasy vector (Promega). Inverse PCR was used to introduce a *BamHI* restriction site and a deletion of appoximately 800 bp in the target genes (32). Inverse PCR products were digested with *BamHI* and ligated to the *BamHI*-digested chloramphenicol resistance (Cm^r) cassette of pAV35 (33). After sequencing, the resulting constructs were electroporated into different *C. jejuni* strains and recombinants were selected on MH plates containing chloramphenicol (33). Junction PCR, with primers up- and downstream of the area involved in the homologous recombination, and primers in the Cm^r cassette, was performed to confirm double cross over events and to assess the orientation of the resistance cassette.

Mass spectrometric analysis

Overnight growths from one agar plate were treated as described by Szymanski *et al.* (34) except that we used proteinase K at 60 μg/mL, RNAse A at 200 μg/mL and DNAse I at 100 μg/mL. The *O*-deacylated LOS samples were analyzed by capillary-electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS) as described by St. Michael *et al.* (35)

SDS-PAGE and Western blotting

Fresh overnight cultures of GB11 wild type and mutant strains were harvested in PBS and lysed by sonification. To visualize both protein and LOS fractions, the cell lysates were electrophoresed on 11% SDS-PAGE gels. To visualize the LOS fraction only, cell lysates were treated with proteinase-K (1mg/ml) for 2 hours at 65°C and electrophoresed on 15% SDS-PAGE gels. Gels were silver stained to visualize LOS cores or the gels were blotted onto nitrocellulose. GB11 patient serum was used at a dilution of 1/2500 and peroxidase-conjugated cholera toxin (Sigma) was used at a concentration of 1 μg/ml.

Mouse immunization experiments

This research was approved by the Animal Care and Use Committee, Dokkyo University School of Medicine (approval no. 00-22). Mice were treated according to the Guidelines for the Care and Use of Laboratory Animals of Dokkyo University School of Medicine. Mice lacking GM2/GD2 synthase as well as GM1 and GD1a (36) were immunized intraperitoneally several times at 2-week intervals with 1 mg of each lyophilized *C. jejuni* dissolved in 100 μl *keyhole lympet hemocyanin* solution, mixed with 100 μl complete Freund's adjuvant. One week after the final inoculation, blood samples were taken from the tail vein. Serum IgG antibodies to GM1, GD1a, and asialo-GM1 were measured by an enzyme-linked immunosorbent assay (37). The mean absorbance value for triplicate reference wells without antigen was subtracted from the mean value for triplicate sample wells with the antigen.

Statistical analysis

Statistical analysis was performed with Instat™ (version 2.05a; Graphpad Software, San Diego, CA) using 2x2 contingency tables. Fisher's exact tests were executed and two sided p-values determined. In case of $P < 0.05$, associations were considered significant.

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Supplemental Table 1. *C jejuni* LOS biosynthesis genes and primer sequences used for their detection

Code[a]	Proposed function	Primer sequences[b]
ORF1abcde	Heptosyl transferase I	
ORF2abcde	Lipid A biosynthesis acyltransferase	
ORF3abcde	Two-domain glucosyltransferase	
ORF4abc	ß-1,3-Galactosyltransferase	
ORF5-Iab	ß-1,4-N-Acetylgalactosaminyltransferase	
ORF5-IIb	ß-1,4-N-Acetylgalactosaminyltransferase	5′-TCATTTCTTTATATAAAAGATACTCG-3′ 5′-TTCTTTAATATTTGCTCGATTACAGG-3′
ORF6abc	ß-1,3-Galactosyltransferase	
ORF7c (cst-III)	α-2,3-Sialyltransferase	
ORF7ab (cst-II)	α-2,3 or α-2,3/α-2,8-Sialyltransferase	5′-ATGAAAAAAGTTATTATTGCTGGAAATG-3′ 5′-TTATTTTCCTTTGAAATAATGCTTTATTC-3′
ORF8abc	Sialic acid synthetase	
ORF9abc	N-acetylglucosamine 2-epimerase	
ORF10ab	CMP-sialic acid synthetase	
ORF5/10c	ß-1,4-N-Acetylgalactosaminyltransferase, CMP-sialic acid synthetase	
ORF11ab	Putative acetyltransferase	
ORF12abcde	Putative glycosyltransferase	
ORF13abcde	Heptosyltransferase II	
ORF14c	Putative glycosyltransferase	5′-CAACTTTGCAAAATGATTTTATCTATCATT-3′ 5′-ATGCAAATACAACAAAACAATTC-3′
ORF15c	Putative glycosyltransferase	
ORF16c	Hypothetical protein	
ORF19d	Putative galactosyltransferase	5′-AATAGTACAAAAGAACTTACAAAAG-3′ 5′-TAAAAAGTTTTAAATTGATACCCAC-3′
ORF21e	Putative glucose-1-phospate thymidyl transferase	5′-GGCATTATTTTAGCTGGAGG-3′ 5′-GGCAATCTTATAACCCTGCC-3′

[a] The letters a,b,c,d and/or e behind the ORF# refer to the LOS locus classes in which the respective ORF is present.
[b] Primers used as class-specific primers for the determination of the LOS locus class. Forward and reverse primer sequences are given, respectively.

Supplemental Table 2. Assignment of the variants for the lipid A backbone and variable terminal Gal (t-Gal), GalNAc, inner (i-NeuAc) and terminal (t-NeuAc) sialic acids in the *O*-deacylated LOS samples from *C. jejuni* GB11 wild-type and mutant constructs.

Strain	Lipid A variant	i-NeuAc	GalNAc	t-Gal	t-NeuAc	Observed mass	Calculated mass
GB11	GlcN3N-GlcN-PPEtn	+	+	+	-	3231.5	3230.1
	GlcN3N-GlcN-2PPEtn	+	+	+	-	3353.0	3353.1
	GlcN3N-GlcN	+	+	+	+	3399.0	3398.3
	GlcN3N-GlcN3N-PPEtn	+	+	+	-	3456.0	3455.4
	GlcN3N-GlcN-PPEtn	+	+	+	+	3522.0	3521.3
	GlcN3N-GlcN3N-2PPEtn	+	+	+	-	3579.5	3578.5
	GlcN3N-GlcN-2PPEtn	+	+	+	+	3644.5	3644.4
	GlcN3N-GlcN3N-PPEtn	+	+	+	+	3747.5	3746.7
	GlcN3N-GlcN3N-2PPEtn	+	+	+	+	3870.0	3869.8
orf10/orf11 mutant	GlcN3N-GlcN	-	-	-	-	2450.5	2450.4
	GlcN3N-GlcN-PPEtn	-	-	-	-	2574.0	2573.5
	GlcN3N-GlcN-2PPEtn	-	-	-	-	2696.5	2696.5
	GlcN3N-GlcN-PPEtn	-	+	-	-	2777.0	2776.7
	GlcN3N-GlcN3N-PPEtn	-	-	-	-	2798.5	2798.9
	GlcN3N-GlcN-2PPEtn	-	+	-	-	2900.5	2899.7
	GlcN3N-GlcN-PPEtn	-	+	+	-	2939.0	2938.8
	GlcN3N-GlcN3N-PPEtn	-	+	-	-	3002.5	3002.0
	GlcN3N-GlcN-2PPEtn	-	+	+	-	3062.5	3061.9
	GlcN3N-GlcN3N-2PPEtn	-	+	-	-	3126.0	3125.1
	GlcN3N-GlcN3N-PPEtn	-	+	+	-	3164.5	3164.2
orf11 mutant	GlcN3N-GlcN-PPEtn	+	+	+	-	3229.5	3230.1
	GlcN3N-GlcN-2PPEtn	+	+	+	-	3353.0	3353.1
	GlcN3N-GlcN	+	+	+	+	3399.0	3398.3
	GlcN3N-GlcN3N-PPEtn	+	+	+	-	3456.0	3455.4
	GlcN3N-GlcN-PPEtn	+	+	+	+	3521.0	3521.3
	GlcN3N-GlcN3N-2PPEtn	+	+	+	-	3579.5	3578.5
	GlcN3N-GlcN-2PPEtn	+	+	+	+	3644.5	3644.4
	GlcN3N-GlcN3N-PPEtn	+	+	+	+	3747.5	3746.7
	GlcN3N-GlcN3N-2PPEtn	+	+	+	+	3869.0	3869.8
cst-II mutant	GlcN3N-GlcN	-	-	-	-	2449.7	2450.4
	GlcN3N-GlcN-PPEtn	-	-	-	-	2574.8	2573.5
	GlcN3N-GlcN-2PPEtn	-	-	-	-	2696.8	2696.5
	GlcN3N-GlcN-PPEtn	-	+	-	-	2777.2	2776.7
	GlcN3N-GlcN-2PPEtn	-	+	-	-	2900.3	2899.7
	GlcN3N-GlcN-PPEtn	-	+	+	-	2938.5	2938.8
	GlcN3N-GlcN-2PPEtn	-	+	+	-	3063.3	3061.9
	GlcN3N-GlcN3N-PPEtn	-	+	+	-	3165.8	3164.2
	GlcN3N-GlcN3N-2PPEtn	-	+	+	-	3288.3	3287.2

Assigments are from a comparison of the observed and calculated mass (Da) based on proposed LOS structures (Figure 3). Average mass units were used for calculation of molecular mass values based on proposed compositions as follows: Glc/Gal, 162.14; GalNAc, 203.19; Hep, 192.17; KDO, 220.18; GlcN: 161.16; GlcN3N, 160.18; NeuAc, 291.26; phosphate, 79.98; PEtn, 123.05; fatty acid, 226.36.

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Chapter 3 PCR-restriction fragment length polymorphism analysis of *Campylobacter jejuni* genes involved in lipooligosaccharide biosynthesis identifies putative molecular markers for Guillain-Barré syndrome

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CHAPTER 4

Evidence for the acquisition by horizontal exchange of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a Guillain-Barré syndrome patient

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Abstract

Campylobacter jejuni GB11, a strain isolated from a Guillain-Barré syndrome patient, has been shown to be genetically closely related to the completely sequenced strain *C . jejuni* NCTC 11168 by various molecular typing and serotyping methods. However, we observed that the lipooligosaccharide (LOS) biosynthesis genes strongly diverged between GB11 and NCTC 11168. We sequenced the LOS biosynthesis locus of GB11 and found that it was nearly identical to the class "A" LOS locus from the *C. jejuni* HS:19 Penner serotype strain (ATCC 43446). Analysis of the DNA sequencing data showed that an horizontal exchange event involving at least 14.26 kb has occurred in the LOS biosynthesis locus of GB11 between *galE* (Cj1131c in NCTC 11168) and *gmhA* (Cj1149 in NCTC 11168). Mass spectrometry of the GB11 LOS showed that GB11 expressed an LOS outer core that mimics the carbohydrate portion of the gangliosides GM1a and GD1a, similar to *C. jejuni* ATCC 43446. The serum from the GB11 patient was shown to react with the LOS from both GB11 and ATCC 43446 but not from NCTC 11168. These data indicate that the anti-ganglioside response in the GB11 patient was raised against the structures synthesized by the acquired class "A" LOS locus.

The Guillain-Barré syndrome is the most common cause of acute neuromuscular paralysis in countries where poliomyelitis has been eradicated. The Miller Fisher syndrome (MFS) is a rare variant of GBS that involves mostly ocular symptoms. Both GBS and MFS are postinfectious neuropathies and *C. jejuni* gastroenteritis is considered to be the most frequent antecedent infection associated with their development (1). The core oligosaccharides of low-molecular-weight lipooligosaccharides (LOS) of many *C. jejuni* strains have been shown to exhibit molecular mimicry with the carbohydrate moieties of gangliosides (2-4). This molecular mimicry between *C. jejuni* LOS outercore structures and gangliosides has been suggested to act as a trigger for autoimmune mechanisms in the development of GBS (5).

Penner et al. (6) developed a serotyping scheme for *C. jejuni* based on soluble heat-stable (HS) antigens. There are a few reports of over-representation of specific Penner serotypes (HS:19 in Japan and HS:41 in South Africa) in GBS-associated *C. jejuni* isolates (7, 8). However the use of various serotyping and molecular typing methods (AFLP, MLST, PFGE and RAPD) failed to show any clustering among GBS and MFS isolates from The Netherlands and Belgium (9-11). Although no clustering was observed when the GBS and MFS strains were compared with enteritis-only strains, *C. jejuni* GB11, a strain isolated from a GBS patient, was found in the same cluster as *C. jejuni* NCTC 11168 (the "genome strain"). *C. jejuni* GB11 and NCTC 11168 have the same heat stable (HS:2) and heat labile (Lior type 4) serotypes. Other evidence that *C. jejuni* GB11 is genetically close to NCTC 11168 is provided by multiple locus sequence typing (MLST) of seven housekeeping genes and of the *flaA* SVR (short variable region). Based on MLST, both *C. jejuni* NCTC 11168 and GB11 were shown to belong to the ST-21 clonal complex (9) and (http://campylobacter.mlst.net). Also the sequences of the *flaA* SVRs of *C jejuni* NCTC 11168 (GenBank AL139078) and GB11 (GenBank AF354548) are 100% identical (321/321). Because *C. jejuni* GB11 is genetically related to the genome strain NCTC 11168 it would be a good candidate as a GBS "prototype" strain to identify any potential neuropathogenic factor specific to GBS/MFS strains. However, there is no way to prove that *C. jejuni* NCTC 11168 would not cause GBS cases if a large group of people were infected with it and there remains a doubt whether it is really an "absolute" enteritis-only control.

Although GB11 and NCTC 11168 are genetically related we observed that the lipooligosaccharide biosynthesis genes strongly diverged between these two strains. Previous work (12) showed that the *C. jejuni* LOS biosynthesis loci of 11 strains could be grouped in 3 classes (A, B and C) based on the gene contents in the region from ORF Cj1133 (heptosyltransferase I) to Cj1149 (heptosyltransferase II). The sequencing of this region in *C. jejuni* GB11 showed that it has a class "A" LOS biosynthesis locus while NCTC 11168 has a class "C" LOS biosynthesis locus. We extended the sequencing to the regions upstream and downstream of the LOS biosynthesis locus (from Cj1130c to Cj1156) in both GB11 (GenBank AY422197) and ATCC 43446 (GenBank AF167344). The region that goes from Cj1132c to Cj1149c is nearly identical in GB11 and ATCC 43446 with only 6 bp differences over a region of 14,260 bp. The corresponding region in NCTC 11168 has diverged considerably with 4 ORFs that are absent in GB11 and ATCC 43446 while the latter have one ORF unique to them (Figure 1). *C. jejuni* ATCC 43446 is the HS:19 Penner

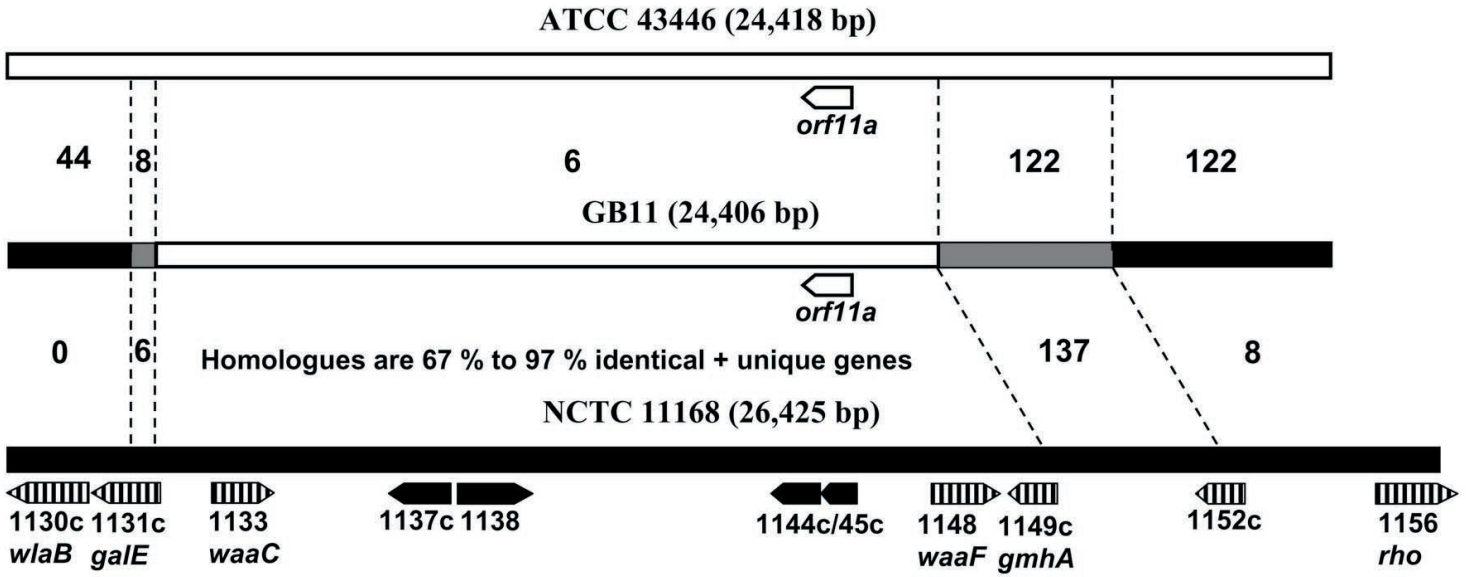

Figure 1. DNA sequence comparison of the region from *Cj1130c* to *Cj1156* in *C. jejuni* GB11 (GenBank AY422197), NCTC 11168 (GenBank AL139077) and ATCC 43446 (GenBank AF167344) strains. The numbers between the loci indicate the number of base differences in each region. The arrows at the bottom indicate the positions of some of the genes in *C. jejuni* 11168 genes (identified by their "Cj" number). The genes in black are specific to *C. jejuni* NCTC 11168 (*Cj1137c* and *Cj1138* encode putative glycosyltransferases while *Cj1144c* and *Cj1145c* encode proteins with unknown functions). The gene in white (*orf11a*, a putative acetyltransferase) is found only in *C. jejuni* GB11 and ATCC 43446. The genes with black and white strips are present in the three strains.

type strain and belongs to the ST-22 clonal complex while the sequence of its *flaA* SVRs is only 79% identical with the corresponding sequence in either GB11 or NCTC 11168 (which are identical). Other molecular typing methods (AFLP, PFGE and RAPD) also showed that *C. jejuni* GB11 is closer to NCTC 11168 than to ATCC 43446 (9, 10) and data not shown). Consequently, the presence of the same LOS locus in both ATCC 43446 and GB11 is probably the result of horizontal gene transfer.

While the GB11 region from Cj1132c to Cj1149c is nearly identical to ATCC 43446, the 5′ (Cj1130c to Cj1131c) and 3′ (Cj1153c to Cj1156) regions are almost identical to NCTC 11168 with 0 difference (over 2,370 bp) and 8 differences (over 4,433 bp), respectively. The near identity between GB11 and NCTC 11168 in these regions and the overall genetic relatedness of these two strains indicate that an "A" class LOS biosynthesis locus was transferred from an HS:19 strain to GB11, rather than in the other direction. The recombination event involved at least 14.26 kb but the exact sites of insertion are unclear. There are "transition" regions that are unique to each strain between the middle region of near identity betweeen GB11 and ATCC 43446 and the 5′ and 3′ regions of near identity between GB11 and NCTC 11168. The high divergence among the three strains in the "transition" regions might be the result of multiple independent cross-over events. This could easily explain the 0.3 kb "transition" region found at the 5′ end of Cj1131c (a UDP-glucose 4-epimerase). The other "transition" region spans four genes (from Cj1149c to Cj1152c) encoding enzymes that are all involved in heptose biosynthesis (www.sanger.ac.uk/Projects/C_jejuni/). This "transition" region is fairly large (3 kb) and GB11 could also have acquired part of it from another strain in a separate recombination event.

Multiple locus sequence typing studies have shown that *C. jejuni* is genetically diverse, with a weakly clonal population structure and that horizontal genetic exchange is common (9, 13). *C. jejuni* is naturally competent (14) and de Boer at al. (15) have shown that genetic exchanges between *C. jejuni* strains can occur during colonization of chickens (a natural reservoir of *Campylobacter*). The strains that belong to the HS:19 Penner type seem to be an exception as they were suggested to comprise a clonal population based on various molecular typing methods (16, 17). De Boer et al. (18) suggested that clonal complexes of *C. jejuni* are genetically preserved by lack of natural transformation. The horizontal transfer of an LOS locus from an HS:19 strain to an HS:2 strain (GB11) is thus consistent with the observations that genetic exchange is common in *C. jejuni* although an HS:19 strain is more likely to act as a donor rather than as a recipient. The near sequence identity between the ATCC 43446 and GB11 LOS locus does not demonstrate that the ATCC 43446 was necessarily the HS:19 donor but is a reflection of the high homogeneity among all HS:19 strains. The LOS biosynthesis locus has been sequenced in four HS:19 strains (GenBank accession numbers AY297047, AF130984, AF167345 and AF167344) and they share above 99% DNA sequence identity.

We used capillary-electrophoresis coupled with electrospray mass spectrometry to obtain data on the LOS structure of GB11 (data not shown). The O-deacylated LOS of GB11 gave an array of masses due to *N*-linked fatty acid and phosphate variability as observed previously with other *C. jejuni* samples (19). The mass profile of the GB11 LOS was the same as the ones

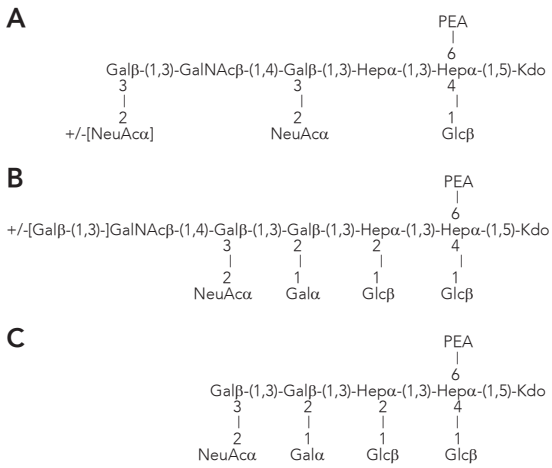

Figure 2. LOS outer core structures expressed by the *C. jejuni* strains studied in this work (2-4, 22).
A: *C. jejuni* ATCC 43432 (HS:4 type strain), ATCC 43446 (HS:19 type strain) and GB11. In this structure the terminal Neu5Ac is variable giving a GD1a mimic when present, and a GM1a mimic when absent;
B: *C. jejuni* NCTC 11168 (the terminal Gal is variable);
C: *C. jejuni* ATCC 43430 (HS:2 type strain).

observed for ATCC 43432 (HS:4 type strain) and ATCC 43446 (HS:19 type strain), two strains which express a mixture of GM1a and GD1a mimics (Figure 2) and have a class "A" LOS locus.

The serum of the GB11 patient was previously shown to react with the LOS of *C. jejuni* GB11 (20). It showed no reaction with the LOS from NCTC 11168 and a strong reaction with the LOS from ATCC 43446 (Figure 3) which is consistent with GB11 having an LOS locus similar to ATCC 43446 (class "A" locus) rather than to NCTC 11168 (class "C" locus). The GB11 serum also reacted with the LOS of ATCC 43432 (HS:4), which is another strain that has a class "A" locus and an LOS structure identical to ATCC 43446 and GB11 (2). The GB11 serum had no reaction with the ATCC 43430 (HS:2) strain which has a "C" class locus and an LOS structure related to the one observed in NCTC 11168, although it is more truncated (3). Some studies have shown that HS:19 strains are more commonly associated with GBS than other serotypes (7) and it is tempting to speculate that GB11 would have acquired the ability to cause GBS following the acquisition of the class "A" locus present in HS:19 strains. Nachamkin et al. (21) has observed a strong association of three class "A" genes (*cstII*, *cgtA* and *cgtB*) with *C. jejuni* strains isolated from GBS patients. However, more GBS and MFS *C. jejuni* strains will have to be analyzed to demonstrate a statistically significant association of the class "A" LOS biosynthesis locus with postinfectious neuropathies. In any case, the serum reactivity of the GB11 patient against the GB11 LOS and gangliosides (20) certainly substantiates the need for further studies of this LOS locus in other GBS and MFS associated strains.

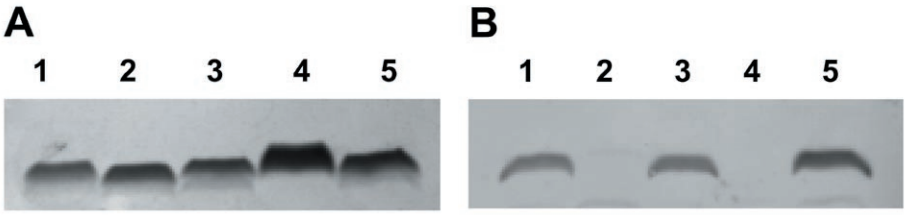

Figure 3. Specific immunodetection of the *C.jejuni* GB11 and ATCC 43446 (HS:19 type strain) lipooligosaccharide by the serum from the GB11 patient. *C. jejuni* ATCC 43432 (HS:4 type strain) is another example of positive reaction and ATCC 43430 (HS:2 type strain) of negative reaction. Proteinase K treated preparations of *C. jejuni* GB11 (lane 1), ATCC 43430 (lane 2), ATCC 43432 (lane 3), NCTC 11168 (lane 4) and ATCC 43446 (lane 5) were electrophoresed on 16.5 % deoxycholate-PAGE.
Panel **A**: the gel was stained using silver stain.
Panel **B**: the LOS was transferred to a PVDF membrane. The membrane was then probed using the GB11 patient serum diluted 1/5,000. The secondary antibody was alkaline phosphatase-conjugated rabbit anti-human IgG and the detection is with NBT/BCIP.

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CHAPTER 5

Co-infection with two different *Campylobacter jejuni* strains in a patient with the Guillain-Barré syndrome

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Abstract

Campylobacter jejuni is the predominant cause of antecedent infection in Guillain-Barré syndrome (GBS) or Miller Fisher syndrome (MFS). *C. jejuni* probably triggers GBS or MFS through molecular mimicry between bacterial sialylated lipo-oligosaccharides (LOS) and gangliosides in peripheral nerve tissue. We investigated whether co-infections with multiple *C. jejuni* strains occur in GBS or MFS patients and we further characterized these strains. PFGE analysis of 83 *C. jejuni* isolates from single primary colonies from stool cultures of 13 patients with GBS or MFS revealed co-infection with two different strains in one patient (8%). We showed that only strain GB5.1 contained an LOS biosynthesis gene locus that is associated with neuropathy. The patient serum strongly reacted with the LOS of strain GB5.1 and not with the LOS of strain GB5.2. Mass spectrometry revealed that both strains expressed a non-sialylated outer core structure in their LOS. The patient serum contained anti-asialo-GM2 antibodies that cross-reacted with the LOS of strain GB5.1. This study demonstrates that co-infection with multiple *C. jejuni* strains occurs in GBS patients. Consequently, not all *C. jejuni* strains isolated from the faeces of a GBS patient are involved in the pathogenesis of GBS per sé. Furthermore, this is the first report in which cross-reactivity of antibodies to asialo-GM2 and to the LOS of a *C. jejuni* strain from a GBS patient has been demonstrated. This finding suggests that molecular mimicry with non-sialylated structures may also be involved in the pathogenesis of GBS.

Introduction

Campylobacter jejuni is not only the most frequent cause of bacterial gastroenteritis, but also the predominant cause of antecedent infection in acute post-infectious neuropathies such as the Guillain-Barré syndrome (GBS) and its variant the Miller Fisher syndrome (MFS) (1). Many studies have provided strong evidence for molecular mimicry as causative mechanism in the development of GBS (reviewed in (2) and (3)). Molecular mimicry refers to a structural resemblance of microbial agents with host structures. *C. jejuni* probably triggers GBS or MFS through molecular mimicry between lipo-oligosaccharides (LOS) in the bacterial cell wall and gangliosides in peripheral nerve tissue (3). Serum anti-ganglioside antibodies that cross-react with *C. jejuni* LOS are present in the majority of *Campylobacter*-associated GBS/MFS patients (3). Various ganglioside-like structures have been identified in the LOS fraction of many different *C. jejuni* strains (4). Accordingly, *C. jejuni*-infected GBS patients have antibodies against a variety of gangliosides and the specificity varies widely between patients. It has been demonstrated that the structure of the LOS fraction is related to the anti-ganglioside specificity and clinical features (5). *C. jejuni* strains from GBS/MFS patients more frequently express ganglioside mimics than strains from patients with enteritis without neurological symptoms (5). However, these latter strains can also harbour ganglioside mimics in their LOS, indicating that ganglioside mimicry per se is not sufficient to induce GBS and that additional pathogen- and/or host-related factors are important as well. Thus, a detailed analysis of GBS/MFS-associated *C. jejuni* strains may lead to the identification of potentially neuropathogenic factors in *C. jejuni* that are involved in the pathogenesis of GBS.

It has been shown that chickens, which are considered to be a major source of *C. jejuni* that infect humans, are often colonized with three or more different *Campylobacter* strains (6). At the retail level, Schouls et al. demonstrated that approximately 30% of raw meat and poultry samples is contaminated with multiple types of *Campylobacter* (7). In contrast, Richardson et al. report that only 7.5% of positive human fecal samples contain multiple *Campylobacter* strains, indicating that the majority of humans are probably infected with one *Campylobacter* strain (8). We are not aware of studies that assessed the presence of co-infections with multiple *Campylobacter* strains in GBS patients. If co-infections exist, then the mere isolation of a *Campylobacter* strain from a GBS patient per se, does not imply its direct involvement in the pathogenesis of the disease. Therefore, the objectives of this study were to assess whether GBS/MFS patients might be co-infected with multiple *C. jejuni* strains and to further characterize these strains.

Materials and methods

Bacterial strains

Starting in 1994, stools from patients presenting with GBS or MFS were cultured using a variety of sensitive and selective techniques (9). From stool samples of 12 patients with GBS and 1 patient with MFS, multiple *C. jejuni* colonies were subcultured and stored separately. We thus obtained 2 to 20 primary isolates per patient. Of most patients, clinical and serological data as well as serum samples were available.

Pulsed field gel electrophoresis

PFGE was performed as described previously (10). In short, samples of genomic DNA extracted from overnight cultures were digested with *SmaI*. Electrophoresis was performed in 1% SeaKem agarose in 0.5x TBE using a BioRad CHEF mapper, programmed in the auto-algorithm mode (run time 19 h, switch time 6.75-25 sec). Gels were stained with ethidium bromide for 15 min, destained in distilled water for 1 h, and photographed under UV radiation. The gels were inspected visually by two different investigators.

Determination of the LOS locus class by PCR

Genes involved in the biosynthesis of LOS are clustered in the LOS biosynthesis locus. The class of LOS locus was determined by PCR tests using primer sets specific for LOS locus class A/B, B, C, D and E (11). PCR assays were performed using a Biomed Thermocycler 60 with a program consisting of 40 cycles of 1 minute at 94°C, 1 minute at 52°C, and 2 minutes at 74°C. Per reaction, approximately 50 ng of template DNA was used in a buffer system consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM of each of the deoxyribonucleotide triphosphates, and 0.2 U Super Taq polymerase. For some of the amplifications timing needed to be adjusted.

Immunoblot analysis

Fresh overnight cultures were harvested in PBS and lysed by sonification. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 11% polyacrylamide gels. To visualize the LOS fraction only, cell lysates were treated with proteinase-K (1mg/ml) for 2 hours at 65°C and electrophoresed on 15% polyacrylamide gels. After electrophoresis, proteins and LOS were stained using silver stain or transferred onto polyvinylidene difluoride (PVDF) membranes. The membrane was then probed using serum, diluted 1/2,000, from the GB5-infected patient. The secondary antibody was alkaline phosphatase-conjugated $F(ab')_2$ fragment rabbit anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., PA), and the detection was with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP).

Mass spectrometry analysis

Overnight growths from one agar plate were treated as described by Szymanski et al. (12) except that we used proteinase K at 60 mg/mL, RNAse A at 200 mg/mL and DNAse I at 100 mg/mL. The *O*-deacylated LOS samples were analyzed by capillary-electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS) as described by St. Michael et al. (13).

ELISA

Patient serum was tested for the presence of IgM and IgG anti-glycolipid antibodies that are cross-reactive with *Campylobacter* LOS using inhibition ELISA as described previously (14). In short, the serum was preincubated with various concentrations of Campylobacter LOS. After centrifugation, residual IgM and IgG antibody activity against glycolipids was measured in an ELISA. Several sera from GBS patients and LOS from *C. jejuni* strains from GBS patients and enteritis controls were used in control studies.

DNA sequencing

DNA sequencing was performed using an Applied Biosystems model 373 automated DNA sequencer and the manufacturer's cycle sequencing kit.

Results

PFGE analysis of C. jejuni isolates from single colonies from stool cultures of GBS or MFS patients

A total of 83 C. jejuni isolates from the stool cultures of 13 GBS/MFS patients, were analyzed by PFGE. Strains from all 13 patients could be distinguished on the basis of variation in the PFGE banding pattern. Two distinct PFGE types were found in the samples of 1/13 patients (8%), indicating that this patient had been infected with at least two different C. jejuni strains (Fig. 1). 12/20 isolates of this patient were PFGE type 1 and 8/20 isolates were PFGE type 2.

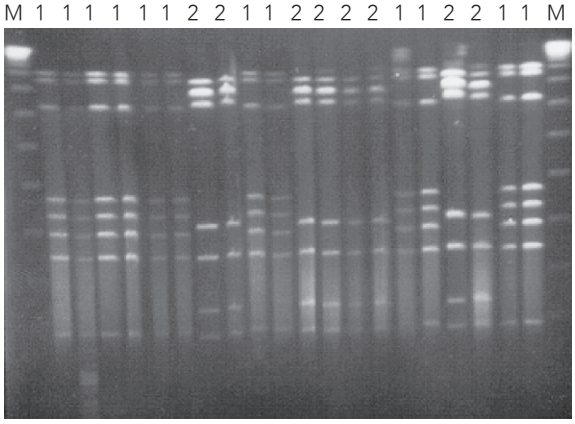

Figure 1. PFGE patterns of twenty *C. jejuni* colonies isolated from one GBS patient (GB5). The two different PFGE types are indicated as 1 and 2. M, molecular size marker.

Characterization of two different C. jejuni strains isolated from a GBS patient

Determination of the LOS locus class

Of both strains (GB5.1 and GB5.2), three randomly selected isolates were subjected to further analyses. Using primers that amplify genes specific for a certain LOS locus, we determined that the three isolates of strain GB5.1 had a class B LOS locus, whereas the isolates of strain GB5.2 had a class E locus.

Immunoblot analysis

We performed immunoblots with the serum of patient GB5. The reactivity of this serum against the protein fraction was comparable for both strains (Fig. 2a). The patient serum showed a strong reactivity against the LOS of strain GB5.1, but it did not react with the LOS of strain GB5.2 (Fig. 2a,c). Control studies demonstrated the specificity of the serum antibodies to LOS from strain GB5.1 since no antibody reactivity was found to LOS of two GM1-mimicking *C. jejuni* strains, GB11 and NCTC11168 (Fig. 2). GB11 was isolated from a GBS patient with anti-GM1 antibodies, NCTC11168 is the "genome strain", originally isolated from an enteritis patient. In

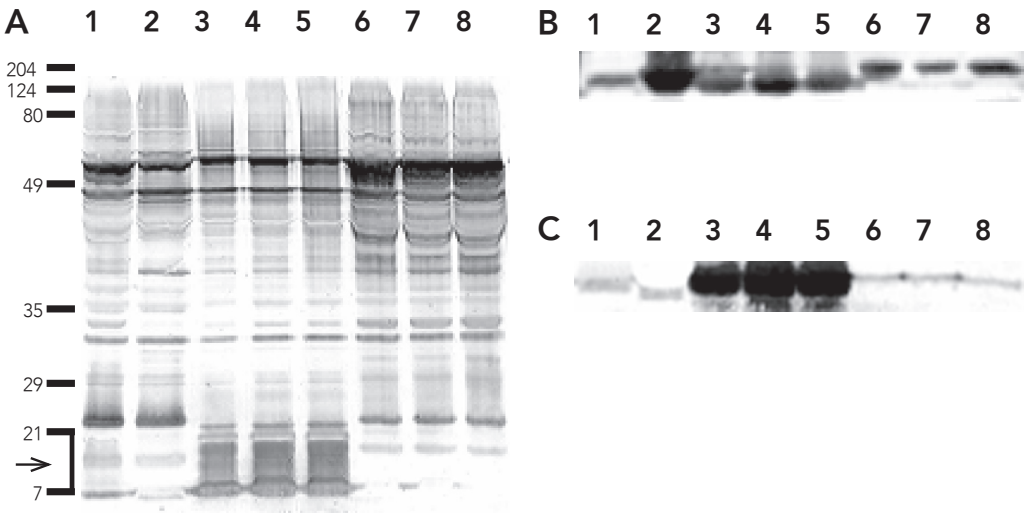

Figure 2. Immunoblot analysis of the two PFGE types, GB5.1 and GB5.2, and two control *C.jejuni* strains, GB11 and NCTC11168, with GB5 patient serum. GB11 was isolated from a GBS patient and expresses GM1-like LOS. NCTC11168, the genome strain, was originally isolated from a patient with enteritis and also expresses GM1-like LOS. Lysates of GB11 (lane 1), NCTC11168 (lane 2), three isolates of GB5.1 (lanes 3-5) and GB5.2 (lanes 6-8) were electrophoresed on an 11% polyacrylamide gel. The gel contents were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with 1:2000 diluted GB5 patient serum. The secondary antibody was alkaline phosphatase-conjugated F(ab')2 fragment rabbit anti-human IgG , the detection was with NBT-BCIP.
(**A**) Immunoblot with whole cell lysates. The arrow and vertical line indicate the region with the LOS reactivity.
(**B**) Silver stained polyacrylamide gel with purified LOS. Note the difference in molecular weight between the LOS of both PFGE types.
(**C**) Immunoblot with purified LOS.

addition, LOS of strain GB5.1 and GB5.2 were not recognized by serum from a GBS patient with anti-GM1 antibodies, from whom the GM1-mimicking control strain GB11 was isolated (results not shown).

Mass spectrometry analysis

We used capillary electrophoresis coupled with electrospray mass spectrometry to determine whether the GB5.1 and GB5.2 strains contain ganglioside-like structures in their LOS (13). This analysis was performed on one isolate of each strain. The mass spectrometry analysis revealed that neither of the two strains contained sialic acid in their LOS (data not shown). Although the mass spectrometry data did not allow us to determine the exact structure of the GB5.1 and GB5.2 outer cores, the observed masses were consistent with GB5.1 LOS containing 3 Hex, 1 HexNAc, 2 Hep, 2 KDO and Lipid A and with GB5.2 LOS containing 3 Hex, 3 HexNAc, 1 QuiNAc, 2 Hep, 2 KDO and Lipid A. The GB5.1 LOS structure, in contrast to GB5.2 LOS, thus resembled asialo-GM2.

Determination of antibodies to glycolipids in patient serum by ELISA

The serum from patient GB5 was tested for IgM and IgG antibodies to glycolipids in ELISA. No IgM or IgG antibodies to GM1, GD1a or GQ1b were detected. However, we found high IgM activity to asialo-GM2 (1:800) and asialo-GM1 (1:800), low IgM titer to GM2 (1:100) and

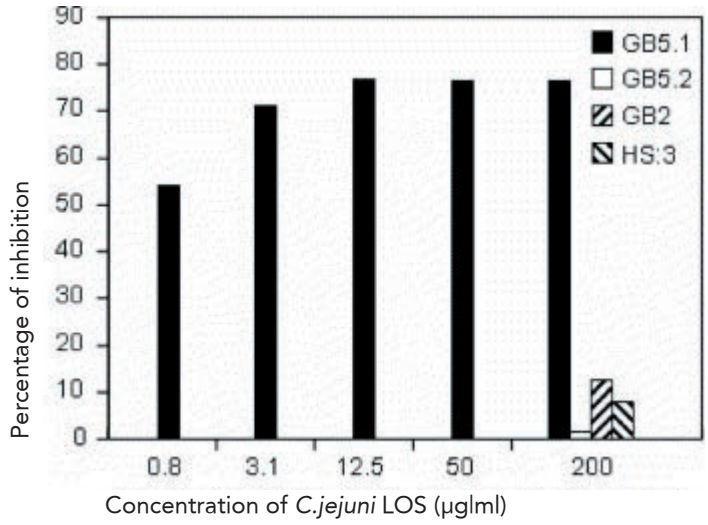

Figure 3. Inibition of IgM activity to asialo-GM2 in serum from patient GB5 by LOS from *C.jejuni* strains. Serum from patient GB5 contained high titer IgM activity to asialo-GM2 (1:800) and asialo-GM1 (1:800) and low IgM titer to GM2 (1:100) in ELISA. Pre-incubation of this serum with LOS from *C. jejuni* GB5.1 (0.8 – 200 µg/ml) showed a dose-dependent inhibition of IgM activity to asialo-GM2 (Fig.3), but not of IgM activity to asialo-GM1 and GM2 (data not shown). Anti-asialo-GM2 IgM was not inhibited by 200 µg/ml of LOS from *C.jejuni* GB5.2, GB2 (which contains mimicry to GM1) (C. Ang, unpublished results) or HS:3 (without ganglioside-mimicry) (15).

low titer of IgG to GM2 (1:100) and asialo-GM1 (1:800). Pre-incubation of this serum with LOS from *C. jejuni* GB5.1 (0.8 – 200 µg/ml) showed a dose-dependent inhibition of IgM activity to asialo-GM2 (Fig.3), but not of IgM and IgG activity to asialo-GM1 and GM2 (data not shown). Anti-asialo-GM2 IgM activity was not inhibited by preincubation with 200 µg/ml of LOS from *C. jejuni* GB5.2 (Fig. 3), indicating that these antibodies were induced by the *C. jejuni* GB5.1 and not by the GB5.2 strain. In further control experiments it was demonstrated that the IgM to asialo-GM2 in this serum did not cross-react with the *C. jejuni* strains GB2 and GB11 (which both contain mimicry to GM1) or HS:3 (without ganglioside-mimicry) (Fig 3) (15). Other control experiments using the inhibition ELISA showed that anti-GM1 IgM in serum from another GBS patient did not bind to LOS from GB5.1 and GB5.2 (data not shown).

DNA sequencing and sequence analysis

Analysis of the DNA sequence of the LOS biosynthesis locus of one GB5.1 isolate (12.4 kb, GenBank accession number AY854153) confirmed that GB5.1 has all the genes necessary for the synthesis of ganglioside-like structures, including *cgtA* (encoding a β-1,4-*N*-acetylgalactosaminyltransferase), *cgtB* (encoding a β-1,3-galactosyltransferase) and *cst-II* (encoding a bi-functional α-2,3/8-sialyltransferase), However, we observed a single base deletion (a missing T) in *cgtB* which would cause a premature translation stop and result in the expression of an inactive CgtB. In addition, sequence analysis showed a single base deletion in the heterogeneous A-tract of the *cst-II* gene which probably leads to an inactive Cst-II as well.

Discussion

We have demonstrated that a co-infection with multiple *C. jejuni* strains occurs occasionally in GBS patients. Although characterization of two strains isolated from one GBS patient revealed that both strains failed to express ganglioside-like structures in their LOS, the patient serum contained anti-glycolipid antibodies cross-reactive with the LOS of only one of the strains.

We found that one of thirteen (8%) GBS and MFS patients had been infected with two *C. jejuni* strains. Although the observed frequency is based on a limited number of GBS patients, it is in agreement with data reported in gastroenteritis patients (7.5%) (8). However, it is possible that the observed frequency of co-infections in GBS patients is underestimated. Twenty primary isolates from this patient had been stored and analyzed. For all other patients, fewer colonies were available for PFGE analysis (range 2-15). Therefore, we cannot exclude that other GBS patients were infected with multiple *C. jejuni* strains as well. In addition, the resolution of PFGE is limited and other, more sensitive, typing procedures might yield higher numbers of (sub)types. The mean time of *Campylobacter* excretion after onset of diarrhoea is 16 days (16) and the onset of neurological symptoms is usually 10-20 days after onset of diarrhoea. Therefore, a co-infection may also be missed if one of the strains is eradicated from the intestinal tract at the time of onset of neurological symptoms. Furthermore, *Campylobacter* serology does not

discriminate between infections with single or multiple strains.

The expression of ganglioside-like structures in the *C. jejuni* LOS is considered to be a crucial factor for the induction of GBS. Genes that are necessary for the biosynthesis of ganglioside-like LOS structures have only been detected in the A, B and C classes of the LOS locus (11, 17), whereas seven different classes of the LOS biosynthesis locus (A to G) have been described so far (18). Previously, we also found that a class A locus is more frequently found in GBS-associated strains, whereas MFS-associated strains contained a class B locus. A class E locus is predominantly found in strains associated with uncomplicated enteritis (19). Therefore, the detection of a class B LOS locus in strain GB5.1 and a class E locus in strain GB5.2. suggests that strain GB5.1 is more likely to be involved in the pathogenesis of GBS. This conclusion is concordant with the observations in the immunoblot analysis. The patient serum strongly reacted with the LOS of strain GB5.1 and not with the LOS of strain GB5.2. So far, we have only observed such strong reactivity against *Campylobacter* LOS in immunoblots with serum from GBS patients (P. Godschalk, unpublished results).

The presence of a class B LOS locus in strain GB5.1 does not imply that this strain really expresses ganglioside-like structures in its LOS. Several mechanisms, such as phase variation, may result in the inactivation of genes that are crucial for the biosynthesis of ganglioside-like LOS (17). Analysis of the LOS structures with mass spectrometry revealed that neither of the two strains contained sialic acid in its LOS. Sialic acid is an essential component of ganglioside-like structures. The strong reactivity of the patient serum against *C. jejuni* LOS without sialylated ganglioside-like structures seems to be in contrast with the current view that sialylated LOS is necessary for the induction of GBS (11). In order to further investigate this apparent inconsistency, we determined the presence of antibodies to glycolipids in the serum from patient GB5. We demonstrated a strong IgM response to asialo-GM2 that could be blocked by LOS from strain GB5.1. These findings indicate that strain GB5.1 induced cross-reactive IgM antibodies to asialo-GM2, which may have played a role in damaging peripheral nerves. Furthermore, the presence of cross-reactive antibodies suggests that GB5.1 expresses asialo-GM2-like LOS, which is concordant with the observed masses in the mass spectrometry analysis. Heterologous infections can alter the immune reactivity towards the individual pathogens involved. Therefore, we cannot exclude the possibility that the immune reactivity against strain GB5.2 had been suppressed due to the co-infection and would have been present in absence of strain GB5.1.

This is the first report in which cross-reactivity of antibodies to asialo-GM2 and to an LOS of a *C. jejuni* strain from a GBS patient has been demonstrated. Molecular mimicry and the induction of cross-reactive antibodies are considered to be a crucial factor in the pathogeneses of GBS, based on accumulating evidence from biochemical, serological, and animal studies (3). Thus far, however, the reported molecular mimicry is with gangliosides. Cross-reactive antibodies to asialo-GM2 and LOS from strain GB5.1 were not identified in the sera from GBS patients tested in control studies, although these may be present in another subgroup of patients. Further studies are needed to demonstrate the pathogenicity of anti-asialo-GM2 antibodies. Alternatively, mechanisms other than molecular mimicry may have played a role in this patient.

Strain GB5.1 has a class B LOS biosynthesis locus which contains all genes that are necessary for the synthesis of ganglioside-like structures (18). However, we found that this strain does not express these structures in its LOS. This can be explained by the presence of a single base deletion in the *cgtB* gene which would cause a premature translation stop and result in the expression of an inactive CgtB. If CgtB is inactive, the terminal Gal residue cannot be added and Cst-II, the sialyltransferase, does not have an acceptor for the sialic acid molecule (17). This deletion may thus result in the expression of an asialo-GM2 LOS outer core structure even if strain GB5.1 contains a class B LOS locus. We also detected a single base deletion in the heterogeneous A-tract of the *cst-II* gene which probably leads to an inactive *Cst-II*.

The detection of abovementioned single base deletions raises the question whether it is possible that these deletions occurred during infection. If so, then the initial expression of ganglioside-like LOS may have induced GBS in this patient. Although we do not consider it very likely that both deletions occurred simultaneously during infection, we cannot completely exclude this possibility. In addition, the antibody response of the patient is concordant with an asialo-GM2-like LOS and not with a sialylated ganglioside-like LOS. It is also possible that GBS was induced by another, non-cultured *Campylobacter* strain or any other microorganism expressing ganglioside-mimicking structures, although antibodies to GM1, GD1a and GQ1b were not detected in serum from this patient. Again, the antibodies that are cross-reactive with the GB5.1 LOS favour the hypothesis that in this patient, GBS was caused by the GB5.1 strain without ganglioside mimicry.

We demonstrated that co-infection with multiple *C. jejuni* strains also occurs in GBS patients. Thus, a *C. jejuni* strain isolated from the faeces of a GBS patient is not necessarily involved in the pathogenesis of GBS. This finding may have implications for the interpretation of typing results of GBS-associated *C. jejuni* strains and the search for potential neuropathogenic determinants. In addition, one should consider analysing multiple colonies from a primary culture of the faeces of a GBS patient. Furthermore, our data suggest that molecular mimicry with structures other than sialylated gangliosides may also trigger GBS. This observation is new and adds to the current knowledge of the pathogenesis of GBS.

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CHAPTER 6 Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barré and Miller Fisher syndrome

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Abstract

Molecular mimicry between lipooligosaccharides (LOS) of *Campylobacter jejuni* and gangliosides in peripheral nerves plays a crucial role in the pathogenesis of *C. jejuni*-related Guillain-Barré syndrome (GBS). We have analyzed the LOS outer core structures of 26 *C. jejuni* strains associated with GBS and its variant the Miller Fisher syndrome (MFS) by capillary-electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS). Sixteen out of 22 (73%) GBS-associated and all 4 (100%) MFS-associated strains expressed LOS with ganglioside mimics. GM1a was the most prevalent ganglioside mimic in GBS-associated strains (10/22, 45%) and in 8 of these strains GM1a was found in combination with GD1a mimics. All 7 strains isolated from patients with ophthalmoplegia (GBS or MFS) expressed disialylated (GD3 or GD1c) mimics. Three out of 22 GBS-associated strains (14%) did not express sialylated ganglioside mimics because their LOS locus lacked the genes necessary for sialylation. Three other strains (14%) did not express ganglioside mimicks because of frame-shift mutations in either the *cstII* sialyltransferase gene or in the *cgtB* galactosyltransferase gene. It is not possible to determine if these mutations were already present during the *C. jejuni* infection. This is the first report in which mass spectrometry combined with DNA sequence data was used to infer the LOS outer core structures of a large number of neuropathy-associated *C. jejuni* strains. We conclude that molecular mimicry between gangliosides and *C. jejuni* LOS is the presumable pathogenic mechanism in most cases of *C. jejuni*-related GBS. However, our findings suggest that in some cases other mechanisms may play a role. Further examination of the disease etiology in these patients is mandatory.

Introduction

Gastro-enteritis caused by *Campylobacter jejuni* is the most common infection preceding the Guillain-Barré syndrome (GBS), an acute immune-mediated neuropathy (1, 2). Molecular mimicry between lipooligosaccharides (LOS) in the *C. jejuni* cell wall and gangliosides in peripheral nerves plays a crucial role in the pathogenesis of GBS (3). Gangliosides are membrane glycolipids that are highly enriched in the nervous system. They are composed of a highly variable oligosaccharide core containing one or more sialic acid molecules and a ceramide tail inserted in the cell membrane. Acute phase sera of most patients with *C. jejuni*-associated GBS contain high titers of antibodies to various gangliosides that cross-react with *C. jejuni* LOS (4, 5). The specificity of these anti-ganglioside antibodies relates to specific antecedent infections and different clinical presentations of GBS. For example, anti-GM1 antibodies have been associated with a preceding *Campylobacter* infection and with a severe, pure motor form of GBS (6). The Miller Fisher syndrome (MFS), a variant of GBS with oculomotor weakness and ataxia, is strongly associated with the presence of anti-GQ1b antibodies (7).

Since the first report in 1993, several studies have demonstrated ganglioside-like structures in the LOS outer core of *C. jejuni* strains isolated from GBS and MFS patients (8). Mass spectrometry (MS) and NMR analysis of individual strains has revealed the presence of GM1a, GD3, GD1a and GT1a mimics in GBS-associated strains and of GD3 mimics in MFS-associated strains (9-13). Serological studies on larger collections of isolates have confirmed and extended these findings (5, 14). However, serological assays are not suitable to determine the exact chemical structure of the LOS outer core.

Detailed knowledge of the biosynthesis and structures of LOS outer cores in neuropathy-associated *C. jejuni* isolates may help to further elucidate the role of microbial factors in the pathogenesis of GBS, especially since *C. jejuni* displays considerable structural variation in its LOS outer core. Several genetic mechanisms responsible for this variation have been described (15). First, there is extensive variation in the gene content of the LOS biosynthesis gene locus ("LOS locus"). In addition, variation in homopolymeric tracts, single base deletions, insertions and mutations can lead to gene inactivations or glycosyltransferases with different acceptor specificities, resulting in the expression of different LOS structures. Previously, we analyzed the LOS locus of a collection of Dutch neuropathy-associated and control enteritis *C. jejuni* isolates (16). We found that the class A LOS locus was associated with GBS and the expression of GM1-like structures, whereas the class B LOS locus was associated with MFS and the expression of GQ1b-like structures. The presence of GM1-like and GQ1b-like structures was determined with serological assays and the exact LOS structures were not known. The development of new MS methods combined with serotyping and preliminary genetic knowledge to predict LOS structures allows quick screening of many strains (17). In the current study, we used this method to infer the LOS outer core structures of 26 GBS- and MFS-associated *C. jejuni* strains. Furthermore, we analyzed the genetic mechanisms responsible for the observed variation in these structures and we related the different LOS structures to clinical symptoms in the corresponding patients.

Materials & Methods

C. jejuni strains. Twenty-two GBS-associated and 4 MFS-associated *C. jejuni* isolates were isolated from patients from The Netherlands, Belgium and the Netherlands Antilles between 1991 and 2000 (Table 1). GB13 and GB14, and GB26 and GB27, were cultured from the diarrheal stools of family members of two GBS patients (18). In both families, there was an outbreak of *C. jejuni* enteritis whereas only one family member developed GBS. From both GBS patients, we were unable to culture *C. jejuni*, despite the serological evidence that the GBS patients had also been infected with *C. jejuni*. These paired isolates were found to be highly related by various genotyping methods (19, 20), suggesting that family members had been infected with the same *C. jejuni* strain. The degree of sub-culturing was kept to a minimum, but 6 to 8 passages were necessary for isolation, storage, transport and preparing cells for mass spectrometry analysis.

Determination of the LOS locus class by PCR. The LOS locus class was determined as described previously (16). To distinguish between class D and class F, an additional primer set for the detection of orf17d (specific for class D) was included (21).

Table 1. *C. jejuni* strains and patient characteristics.

Strain	HS serotype[a]	Origin	Patient	GenBank Accession No[b]
GB1	1	The Netherlands	GBS	EF066651
GB2	UT	The Netherlands	GBS	DQ813306
GB3	19	The Netherlands	GBS	DQ906040
GB4	37	The Netherlands	GBS	AY943308
GB5	4, 64	The Netherlands	GBS	AY854153
MF6	4, 64	The Netherlands	MFS	AY422196
MF7	35	The Netherlands	MFS	DQ140270
MF8	23, 36	The Netherlands	MFS	DQ102714
GB11	2	The Netherlands	GBS	AY422197
GB13	2	The Netherlands	enteritis, family GBS	EF101695
GB14	2	The Netherlands	enteritis, family GBS	EF101696
GB15	5, 34	The Netherlands	GBS	AY423554
GB16	13, 66	Belgium	GBS (with ophthalmoplegia)	EF076703
GB17	4, 13, 64	The Netherlands	GBS	EF094857
GB18	19	The Netherlands	GBS	DQ868320
GB19	4, 50	The Netherlands	GBS (with ophthalmoplegia)	DQ357237
MF20	2	The Netherlands	MFS	EF064287
GB21	13, 65	The Netherlands	GBS	EF076704
GB22	13, 64	Netherlands Antilles	GBS	EF091821
GB23	4, 13, 43	The Netherlands	GBS	EF107518
GB24	31	The Netherlands	GBS	AY573819
GB25	2	The Netherlands	GBS (with ophthalmoplegia)	EF064288
GB26	1, 44	The Netherlands	enteritis, family GBS	DQ351737
GB27	1, 44	The Netherlands	enteritis, family GBS	EF095404
GB28	19, 38	Netherlands Antilles	GBS	DQ906041
GB31	13, 50	Netherlands Antilles	GBS	DQ518908

[a] HS = heat stable (Penner serotyping system).
[b] GenBank accession numbers are given for the partial DNA sequences within the LOS locus

Mass spectrometry analysis

Confluent overnight growths from one agar plate (Mueller-Hinton medium) were treated as described by Szymanski et al. except that we used proteinase K at 60 mg/mL, RNase A at 200 mg/mL and DNase I at 100 mg/mL (17). The *O*-deacylated LOS samples were analyzed by capillary-electrophoresis coupled with electrospray ionization mass spectrometry (CE-ESI-MS) as described by St. Michael et al. (22). All CE-ESI-MS and CE-ESI-MS/MS were performed using a crystal Model 310 capillary electrophoresis instrument (ATI Unicam, Boston, MA) coupled to an API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) via a microIon-spray interface.

DNA sequencing

Genomic DNA was isolated using a DNeasy Tissue kit (Qiagen). Long PCR products were generated using an Advantage 2 PCR kit (Clontech Laboratories). The PCR products were sequenced using custom-made primers that were used previously to sequence the LOS locus in multiple strains (15). DNA sequencing was performed using an Applied Biosystems model 373 automated DNA sequencer (Montreal, Canada) and the manufacturer's cycle sequencing kit. See supplementary Table XXVII for additional details.

Cloning and expression of the cst-II gene from C. jejuni GB26

The GB26 *cst-II* gene was amplified using *Pwo* polymerase (Roche Diagnostics, Laval, Canada) and the following primers: CJ-131 (5' CTTAGGAGGT*CATATG*AAAAAAGTTATTATTGC-TGGAAATG 3', 41 mer, *Nde*I site in italics) and CJ-764 (5' TTTAGG*GTCGAC*TCAAAGATTAAA-ATTTTTTGAG 3', 34 mer, *Sal*I site in italics). These two primers amplified the region encoding amino acids 1 to 260 of *cst-II* from *C. jejuni* GB26. The PCR product was digested with *Nde*I and *Sal*I and cloned in pCWori+(-*lacZ*) (23) giving construct CST-125. *E. coli* AD202 containing construct CST-125 was grown in 2 YT medium containing 150 μg/mL ampicillin. The culture was incubated at 37 °C until A_{600} = 0.35, induced with 1 mM IPTG, and then incubated 7 h at 37 °C. The cells were broken using an Avestin C5 Emulsiflex cell disruptor (Avestin, Ottawa, Canada). α-2,3- and α-2,8-sialyltransferase activities were assayed as described previously (15).

Statistical analysis

Differences in frequencies between groups were analyzed with the Fisher's exact test using InStat version 3.0 (Graphpad Software, San Diego, CA). Differences were considered significant at $P < 0.05$ after two-sided testing.

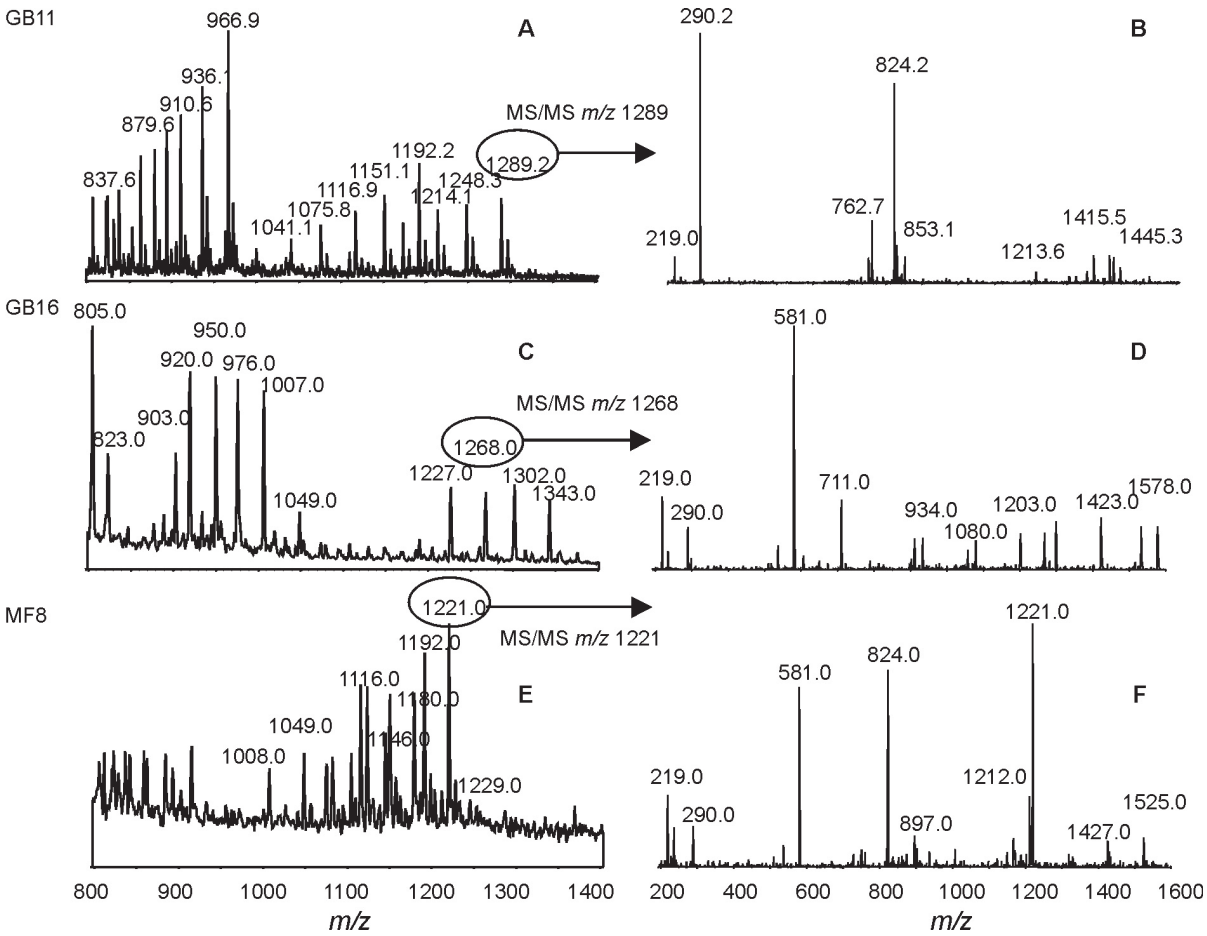

Figure 1. Mass spectrometry analysis of *O*-deacylated LOS from repesentative C. jejuni strains with sialylated LOS outer cores. Panels A and B: C. jejuni GB11, Panels C and D: *C. jejuni* GB16, Panels E and F: C. jejuni MF8. Panels A, C and E show extracted mass spectra from CE-MS. Panels B, D and F show MS/MS of a representative peak from each CE-MS spectrum.

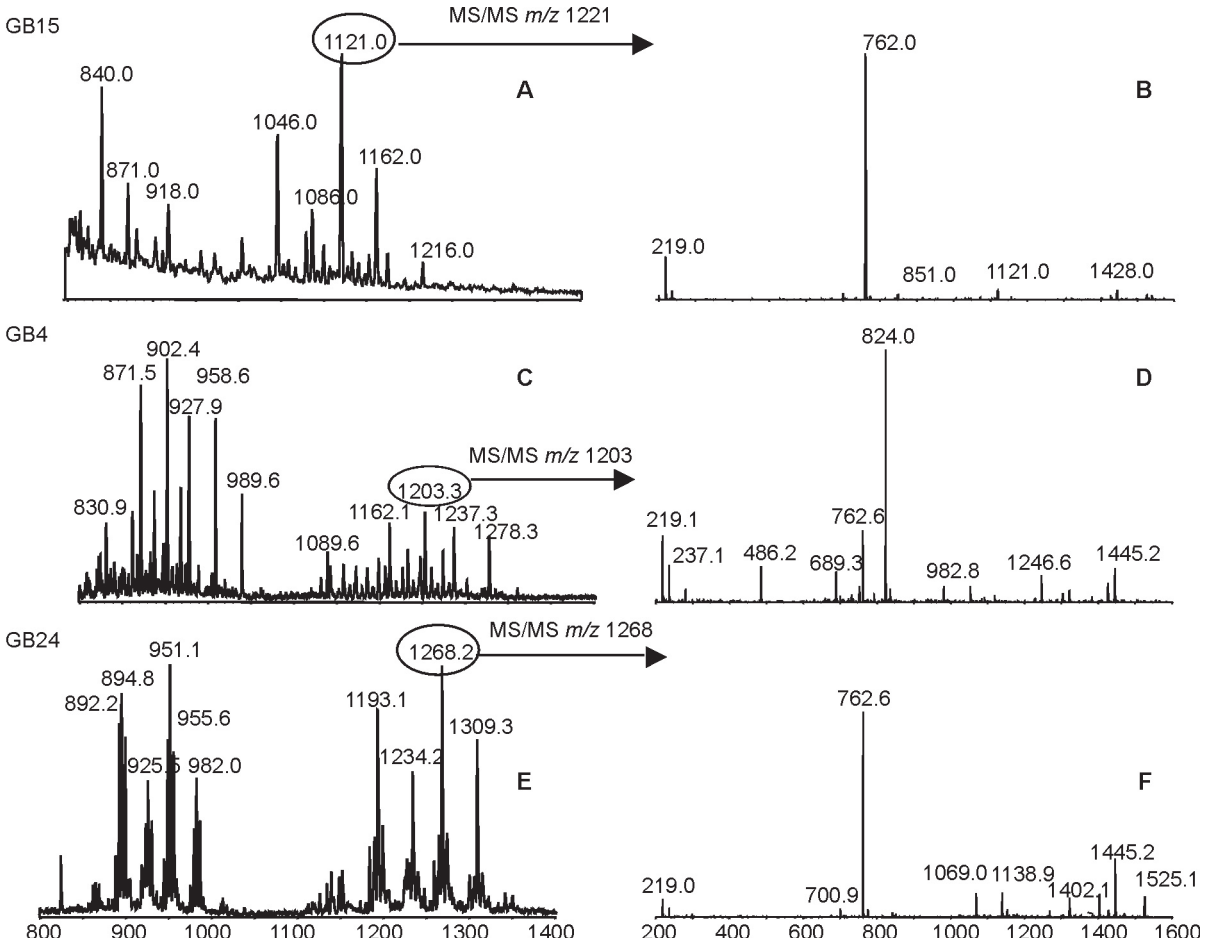

Figure 2. Mass spectrometry analysis of *O*-deacylated LOS from repesentative *C. jejuni* strains with non-sialylated LOS outer cores. Panels A and B: C. jejuni GB15, Panels C and D: C. jejuni GB4, Panels E and F: C. jejuni GB24. Panels A, C and E show show extracted mass spectra from CE-MS. Panels B, D and F show MS/MS of a representative peak from each CE-MS spectrum.

Table 2. Proposed LOS outer core structures expressed by GBS- and MFS-associated strains.

Strain(s)	LOS class	Structure	Ganglioside mimic
GB2, GB3, GB11, GB18, GB21, GB22, GB28, GB31	A	Gal-GalNAc-Gal-HepII-HepI NeuAc Glc	GM1a
		Gal-GalNAc-Gal-HepII-HepI NeuAc NeuAc Glc	GD1a
GB23	A	GalNAc-Gal-HepII-HepI NeuAc Glc	GM2
GB26	A	Gal-GalNAc-Gal-HepII-HepI NeuAc Glc Glc	GM1b
GB27	A	Gal-GalNAc-Gal-HepII-HepI Glc Glc	none (GA1)
GB16, GB19	A	Gal-GalNAc-Gal-HepII-HepI NeuAc Glc Glc NeuAc	GD1c
GB17, GB25	B	Gal-GalNAc-Gal-HepII-HepI Glc Glc	none (GA1)
		Gal-GalNAc-Gal-HepII-HepI NeuAc Glc Glc	GM1b
		Gal-GalNAc-Gal-HepII-HepI NeuAc Glc Glc NeuAc	GD1c
MF6, MF20	B	Gal-GalNAc-Gal-HepII-HepI NeuAc Glc Glc	GM1b
	B	Gal-GalNAc-Gal-HepII-HepI NeuAc Glc Glc NeuAc	GD1c
MF7	B	GalNAc-Gal-Glc-HepII-HepI NeuAc Glc	GM2
		GalNAc-Gal-Glc-HepII-HepI NeuAc Glc NeuAc	GD2
		Gal-Glc-HepII-HepI NeuAc Glc NeuAc	GD3
MF8	B	GalNAc-Gal-Glc-HepII-HepI NeuAc Glc	GM2
		Gal-Glc-HepII-HepI NeuAc Glc NeuAc	GD3
GB5	B	GalNAc-Gal-HepII-HepI Glc Glc	none (GA2) [a]
GB13, GB14	C	Gal-GalNAc-Gal-Gal-HepII-HepI NeuAcGal Glc Glc	GM1a
GB1	C	Gal-Gal-HepII-HepI Gal Glc Glc	none
GB24	K	Hex6 HexNAc1 Hep2	none
GB4	P	Hex3HexNAc3QuiNAc1Hep2 [b]	none
GB15	F	Hex4 HexNAc1 Hep2	none

The brackets indicate the portions of the LOS outer cores that are mimicking gangliosides. The HepI of the inner core in linked to a Kdo residue that is linked to the lipid A portion of the LOS (11).

a The exact structure could not be deduced from the observed masses, but the composition of the sugar residues was consistent with an "asialo-GM2"-like (GA2) structure.

b The observed masses suggest that this LOS structure is related to the LOS outer core structure of strain ATCC 43431 (HS:3 type strain)(26)

Results

Determination of the LOS outer core structures

We used CE-ESI-MS on *O*-deacylated *C. jejuni* LOS to propose LOS outer core structures for the 26 GBS- and MFS-associated isolates (Figures 1 and 2, Table 2, supplementary Tables I to XXVI). The CE-ESI-MS procedure did not provide linkage information but provided information about the sugar composition of the LOS outer core. The glycosyltransferase variants present in the LOS locus of each strain (Table 3) were used to help interpret the data obtained by CE-ESI-MS (see supplementary appendixes A and B). For several strains, we could only determine sugar composition of the LOS outer core and no structure. In classes A and B strains, the presence of a two-domain Cj1135 (glucosyltransferase) suggests that both heptoses are substituted with glucose while the presence of a one-domain Cj1135 suggests that only HepI is substituted with

Table 3. Variants of the glycosyltransferases involved in the synthesis of the LOS outer core structures in *C. jejuni* strains with class A or B LOS locus.[a]

Strain	LOS class	Cj1135	Cj1136	CgtAI	CgtAII	CgtB	Cst-II [b]
GB2	A	One-domain	on	Mono-sialyl.[c]	N/A [d]	Mono-sialyl.	Mono-
GB3	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB5	B	Two-domain	on[e]	Non-sialyl.[f]	Mono/di-sialyl.[g]	off [h]	off
MF6	B	Two-domain	on	Non-sialyl.	Mono/di-sialyl.	Non-sialyl.	Bi-
MF7	B	Two-domain	off	off	Mono/di-sialyl.	off	Bi-
MF8	B	Two-domain	off	off	Mono/di-sialyl.	Mono-sialyl.	Mono-
GB11	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB16	A	Two-domain	on	Non-sialyl.	N/A	Non-sialyl.	Bi-
GB17	B	Two-domain	on	Non-sialyl.	Mono/di-sialyl.	Non-sialyl.	Bi-
GB18	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB19	A	Two-domain	on	Non-sialyl.	N/A	Non-sialyl.	Bi-
MF20	B	Two-domain	on	Non-sialyl.	Mono/di-sialyl.	Non-sialyl.	Bi-
GB21	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB22	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB23	A	One-domain	on	Mono-sialyl.	N/A	off	Mono-
GB25	B	Two-domain	on	Non-sialyl.	Mono/di-sialyl.	Non-sialyl.	Bi-
GB26	A	Two-domain	on	Non-sialyl.	N/A	Non-sialyl.	Mono-
GB27	A	Two-domain	on	Non-sialyl.	N/A	Non-sialyl.	off
GB28	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-
GB31	A	One-domain	on	Mono-sialyl.	N/A	Mono-sialyl.	Mono-

[a] Assignment of the glycosyltransferase variants is based on amino acid sequence comparisons with variants of known specificities (15, 24).
[b] Cst-II variants: mono-: monofunctional, Cst-II has α-2,3-sialyltransferase activity, bi-: bifunctional, Cst-II has both α-2,3-sialyltransferase and α-2,8-sialyltransferase activity.
[c] Mono-sialyl.: the glycosyltransferase is specific for mono-sialylated acceptor.
[d] N/A: Not applicable.
[e] on: indicates that a gene has no frame-shift mutation.
[f] Non-sialyl.: the glycosyltransferase is specific for non-sialylated acceptor.
[g] Mono/di-sialyl.: the glycosyltransferase can use a mono- or a di-sialylated acceptor.
[h] off: indicates that a gene is inactive because of a frame-shift mutation

glucose (24). The extension of the outer core from HepII is proposed for the strains that have an active Cj1136 variant while the two strains (MF7 and MF8) that have an inactive Cj1136 variant due to frame-shift mutations (see GenBank DQ140270 and DQ102714) are proposed to have an outer core extended from the glucose substituted to HepII. Based on our previous observations with strains whose LOS outer core structures were completely determined (24), we propose that the inner galactose is substituted with a sialic acid in the strains that have no glucose on HepII and that have CgtA and CgtB variants that are specific for a sialylated acceptor. We propose that the inner galactose is not substituted with sialic acid in the case of classes A and B strains that have a glucose on HepII, an active Cj1136 variant and CgtA/CgtB variants that are specific for non-sialylated accceptors.

Fifteen different outer core structures were identified among the 26 strains that were analyzed and 14 strains expressed a mixture of at least 2 different outer core structures. It was not possible to quantify the proportions of the different outer core structures because their different sialic acid contents result in different ionization efficiencies, which then have an impact on observed peak intensities. Several strains harboring the same LOS locus expressed different LOS structures. Within the class A strains, 5 different (mixtures of) LOS structures were detected. Clearly, knowledge of the LOS locus class is not sufficient to predict the LOS structure. It is also necessary to sequence the key glycosyltransferases to determine the variants involved and whether they encode complete or truncated products.

Expression of ganglioside mimics in the LOS

Sixteen of 22 (73%) GBS-associated isolates and all 4 (100%) MFS-associated isolates expressed LOS with ganglioside mimics including GM1a, GM1b, GM2, GD1a, GD1c, GD2 and GD3. Ganglioside mimics were only detected in strains with a class A, B or C LOS locus (presence of ganglioside mimics in class A/B/C vs. other classes: 20/23 vs. 0/3, $P<0.01$). In GBS-associated strains, GM1a was the most prevalent ganglioside mimic, present in 10 out of 22 strains (45%). Interestingly, in all 8 GBS strains with a class A LOS locus and GM1a mimicry (36% of all GBS strains), the GM1a mimic was present as part of a GM1a/GD1a mixture (presence of GM1a/GD1a in class A vs. non-A: 8/13 vs. 0/13, $P<0.01$). All 7 strains isolated from patients with MFS or GBS with ophthalmoplegia expressed structures with a terminal di-NeuAc-Gal (GD3 and GD1c), versus only 1/19 other GBS-associated strains (5%, $P<0.01$). These mimics were predominantly found in strains with a class B LOS locus (presence of GD1c or GD3 in class B vs. non-B: 6/7 vs. 2/19, $P<0.01$). Both class A strains with a GD1c mimic were isolated from GBS patients with ophthalmoplegia.

LOS outer core structures without ganglioside mimics

Ganglioside mimics could not be detected in the LOS of 6 out of 22 GBS-associated isolates (27%): GB1, GB4, GB5, GB15, GB24 and GB27 (Table 2). These strains were further analyzed to explain the absence of ganglioside mimics. The class C LOS locus of strain GB1 contains all genes necessary to synthesize sialylated LOS. However, we found a 5-base deletion in the *cst-*

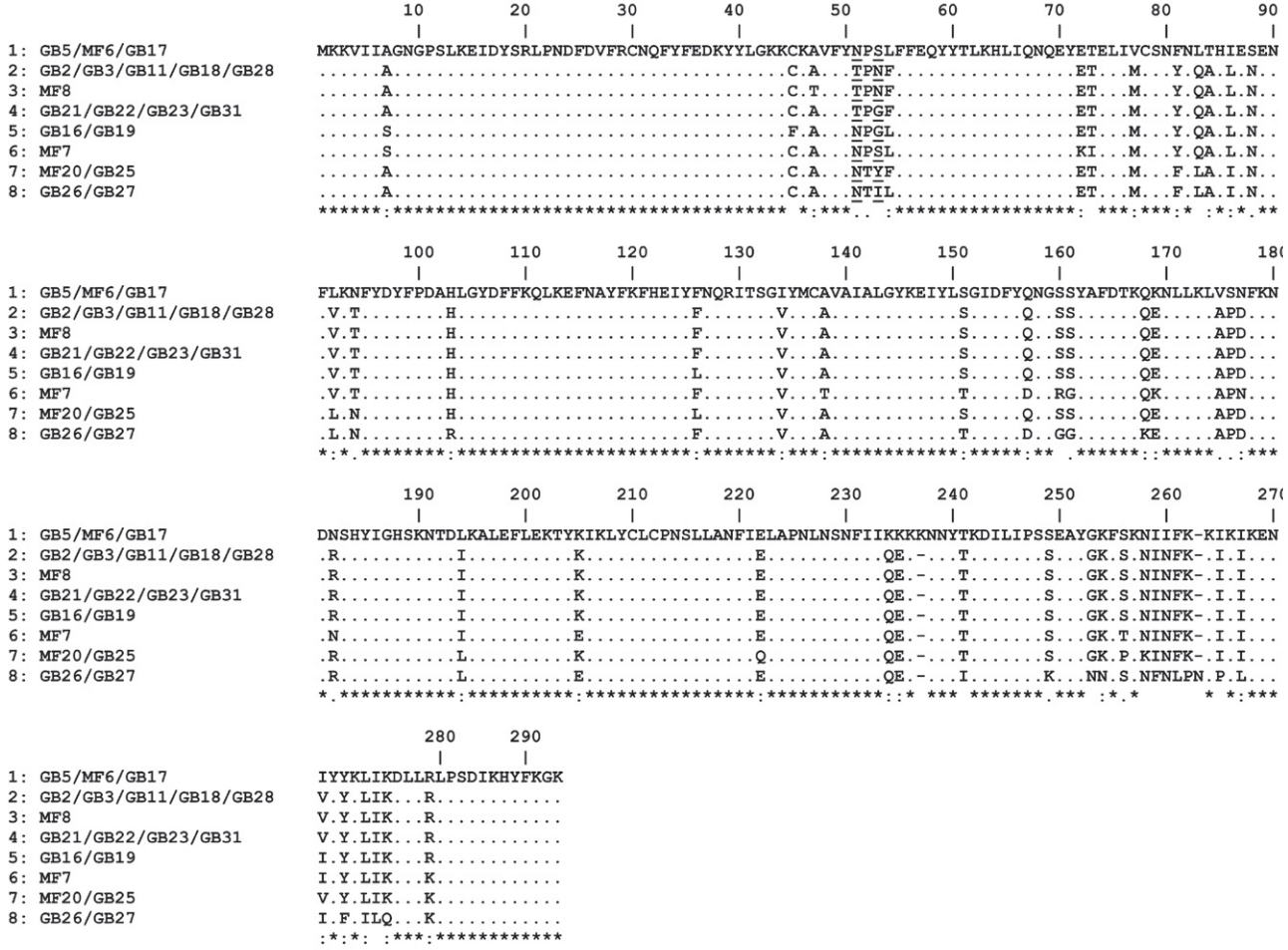

Figure 3. Alignment of the Cst-II sialyltransferase amino acid sequences from the GBS and MFS *C. jejuni* strains. Only variable residues are shown in addition to the consensus sequence. The "*" indicate conserved residues, the ":" indicate strongly similar residues and the "." weakly similar residues. The amino acids that were shown to influence the activity and specificity of Cst-II are underlined (residues 51-53).

III gene of GB1 (GenBank accession number EF066651), resulting in a truncated Cst-III (219 aa instead of 294 aa). This will prevent the transfer of sialic acid and subsequent addition of the terminal GalNAc to the LOS backbone. Strain GB5 also contains a LOS locus (class B) that is essentially capable of directing the synthesis of sialylated LOS, but single base deletions in the *cgtB* and *cst-II* genes result in the expression of a truncated LOS outer core without sialic acid (25). Although GB26 and GB27, isolated from two family members of a GBS patient, were indistinguishable by various pheno- and genotyping methods (16, 20), mass spectrometry revealed that only GB26 expresses sialylated LOS. We detected a poly-G tract in the *cst-II* gene that leads to a frame shift and premature translation stop in GB27 (10-G tract) and mostly a complete translation product in GB26 (9-G tract).

We sequenced the entire LOS biosynthesis locus of GB4, GB15 and GB24 and found that all three strains lack the genes necessary for sialylation of the LOS (GenBank accession numbers AY943308, AY423554 and AY573819, respectively). These three LOS loci do not contain either the sialyltransferase gene (*cst-II* or *cst-III*) or the genes (*neuA*, *neuB* and *neuC*) necessary for the synthesis of sialic acid and its activated donor, CMP-NeuAc. GB15 has a class F LOS locus (24), whereas both GB4 and GB24 contained novel LOS loci (classes P and K, respectively, Parker et al., manuscript in preparation). The exact LOS outer core structures of strains GB4 and GB24 could not be deduced from the mass spectrometry data, but the former structure is related to the LOS outer core of strain ATCC 43431 (HS:3 type strain), which does not contain a ganglioside mimic (26). The mass spectrometry profile suggests that the LOS outer core of GB15 is composed of 4 hexoses and 1 HexNAc. It is possible that the LOS outer core of GB15 mimics a human glycolipid of the globo- or isoglobo- series. However, none of the LOS outer cores of GB4, GB15 and GB24 contains sialic acid, as shown by the absence of the diagnostic ion (*m/z* 290) in CE-MS/MS spectra of *O*-deacylated LOS samples from theses strains (Figure 2). This was further confirmed by precursor ion scan experiments, in which no glycoforms were detected with a precursor ion at *m/z* 290 (data not shown).

Cst-II variants and LOS structure

Polymorphism in the *cst-II* gene determines the extent of sialylation of the LOS (15). Therefore, we determined the correlation between Cst-II variants and LOS outer core structure. We have previously shown that amino acid residues 51 and 53 affect the level of activity and specificity of Cst-II (15). Most of the variants with Asn51 express disialylated LOS outer cores (bifunctional Cst-II; α-2,3- and α-2,8-sialyltransferase activity) while most of the variants with Thr51 express LOS outer cores with only α-2,3-linked sialic acids (monofunctional Cst-II; only α-2,3-sialyltransferase activity). Cst-III, the Cst-II homologue present in class C strains, is always monofunctional and was therefore not analyzed in this study. We detected 8 different variants among the 20 classes A and B neuropathy-associated strains (Figure 3), 5 of which had Asn51. Seven out of 10 strains (70%) with an Asn51 variant expressed disialylated LOS, as opposed to 1 out of 10 strains (10%) with a Thr51 variant (P=0.02). However, the correlation between Cst-II variants and LOS structure was not perfect. MF8 has the Thr51 variant and expresses a mixture of mono- and

di-sialylated LOS outer cores, while GB26 has Asn51 and expresses only mono-sialylated LOS outer core. The LOS loci of MF8 and HS:36 are identical except for a difference in the length of the G-tract in *cgtA* (15). Although speculative, it is possible that a *cgtA* mostly in an "off status" enables Cst-II with very low α-2,8-sialyltransferase activity to add a second sialic acid, since the lack of GalNAc addition preserves the acceptor for Cst-II. We cloned and expressed Cst-II from GB26 and found that it has only α-2,3-sialyltransferase activity (using in vitro assays, supplementary Figure 1) although it has Asn51. Cst-II from GB26 has the sequence that diverges most from the other Cst-II sequences (Figure 3) and it is possible that one (or several) amino acid substitution(s) has(ve) inactivated the α-2,8-sialyltransferase activity. We observed that the Asn51 variant was present in 6/7 class B strains (86%), whereas Thr51 was the most prevalent variant in class A strains (9/13, 69%, *P*=0.06). Likewise, the Asn51 variant was present in 6/7 (86%) of MFS-associated strains and strains associated with GBS and ophthalmoplegia, whereas the Thr51 variant was primarily found in the other GBS-associated strains (9/13, 69%, *P*=0.06).

Discussion

Ganglioside mimicry is considered to be a crucial factor in the pathogenesis of *C. jejuni*-associated GBS (3). Detailed knowledge of the bacterial components mimicking human structures, the genetic mechanisms responsible for the observed variation in these structures and their relation to cross-reactive auto-antibodies and clinical features may provide a better understanding of the role of molecular mimicry in post-infectious neuropathy. For the first time, MS combined with DNA sequence data was used to determine the LOS outer core structures of a large number of neuropathy-associated *C. jejuni* strains. Our data confirm that ganglioside mimicry is the most likely pathogenic mechanism underlying the majority of *C. jejuni*-associated GBS cases, but that in some GBS patients mimicry towards microbial structures other than ganglioside-like LOS or other mechanisms may lead to the neurological damage.

Various ganglioside mimics were found in the LOS of neuropathy-associated strains. GM1a was the most prevalent ganglioside mimic in GBS strains and it was predominantly present in combination with GD1a mimics (36% of all GBS strains). Although GM1a mimics were found in both class A and class C strains, the GM1a/GD1a mixture was only present in strains with a class A LOS locus, which has previously been associated with GBS (16, 27). Because the prevalence of a class A LOS locus in enteritis-associated strains is 14-17% (16, 27), it is expected that a maximum of 14-17% of these strains have the GM1a/GD1a mixture. The high prevalence of a GM1a/GD1a mixture in GBS-associated strains suggests that a cluster or complex of these two ganglioside mimics may be the target antigens in a subgroup of GBS, rather than single ganglioside mimics. This finding is consistent with the results of Koga et al., but in contrast with those of Nachamkin et al., who found that expression of GD1a and not GM1 was associated with GBS (14, 27). Furthermore, our results are in agreement with recent observations that ganglioside complexes are important target antigens in GBS as well as in MFS (28, 29).

MFS and GBS with ophthalmoplegia (GBS/MFS overlap) have been associated with the presence of anti-GQ1b antibodies and with the presence of GQ1b-like LOS as determined with serological assays (5, 7). This association may be explained by the enrichment of GQ1b in the nerves that innervate the oculomotor muscles (30). Up to now, MS analysis has not demonstrated true GQ1b-like structures in *C. jejuni* LOS. The detection of structures with a terminal di-NeuAc-Gal in 7 out of 8 (87%) strains associated with ophthalmoplegia suggests that in these patients, pathogenic antibodies are raised against the disialylated, GD3- or GD1c-like LOS and cross-react with GQ1b in the human nerves.

There are several possible explanations for the observation that 6 GBS-associated strains did not express ganglioside mimics in their LOS. We have previously demonstrated that a GBS patient had been co-infected with two *C. jejuni* strains while only one strain could be linked to GBS (25). In such cases, it is possible that a co-infecting strain, possibly a strain without ganglioside mimics, is isolated from the stool sample and wrongfully regarded as "GBS-associated" strain. This may also have occurred in patients described here related to strains that lacked ganglioside mimics. However, it is also possible that the expression of ganglioside mimics vanished during the infection or culture procedures due to mechanisms such as phase variation or single base mutations or deletions. Strain GB1 did not express ganglioside mimics due to a frame-shift mutation in the *cst-III* gene. It is possible that GBS was induced by a ganglioside-mimicking GB1 strain and that this mutation occurred later in the course of the infection or during laboratory processing. This hypothesis is concordant with the presence of antibodies against both GM1 and asialo-GM1 in the patient serum (5). The same scenario may also apply to strains GB26 and GB27, which had been isolated from two family members of a GBS patient who did not develop neurological symptoms. Both isolates are genetically highly related, indicating that all family members had probably been infected with the same strain. Interestingly, we found that variation in the poly-G tract of the *cst-II* gene was responsible for the lack of ganglioside mimics in the LOS of GB27. In this case, GBS may have been triggered by the ganglioside-mimicking variant of the strain (GB26) and not by the variant without ganglioside mimics (GB27). Strain GB26 expresses an LOS outer core that mimics GM1b which, unfortunately, is not commercially available. Consequently we could not determine if the family member who developped GBS had any anti-GM1b antibodies.

On the other hand, our findings indicate that sometimes, molecular mimicry with non-sialylated LOS may be involved in the pathogenesis of GBS. We demonstrated previously that the GB5 patient serum contains anti-asialo-GM2 antibodies cross-reactive with GB5 LOS, which suggests that GBS was induced by molecular mimicry with *C. jejuni* LOS without ganglioside mimics (25). Other mechanisms, including mimicry with *C. jejuni* structures other than LOS, either sialylated or non-sialylated, or with other microorganisms should also be considered in some cases. Strains GB4, GB15 and GB24 do not express ganglioside mimics because they do not have the genes that are required for sialylation of the LOS. The acute phase patient sera of GB4, GB15 and GB24 did not contain anti-ganglioside antibodies ((5) and M. Kuijf, unpublished data), suggesting a pathogenic mechanism other than ganglioside mimicry. Further investiga-

tions are needed to elucidate the pathogenesis of GBS and the role of *C. jejuni* in these cases.

Genetic polymorphism of *C. jejuni* determines the LOS structure and thereby also the specificity of the anti-ganglioside antibody response and clinical features of GBS (5, 15, 16). Presence of and polymorphism within the *cst-II* gene has been associated with the expression of ganglioside mimics and with clinical features of GBS (31, 32). We found that the Cst-II Asn51 variant was associated with the expression of disialylated LOS and seemed to occur more frequently in class B strains and strains related with clinical symptoms of MFS or GBS with ophthalmoplegia. The Thr51 variant was associated with monosialylated LOS and seemed to occur more frequently in class A strains and in GBS-related strains. These observations suggest that the previously described associations between a class A LOS locus and GBS and class B LOS locus and MFS may be based on the high prevalence of the Thr51 variant in the class A LOS locus and the Asn51 variant in the class B LOS locus (16). Our findings are concordant with the recent reports of Koga et al. (27, 32).

We conclude that the majority of *C. jejuni* strains isolated from GBS or MFS patients express single or multiple ganglioside mimics in their LOS. However, a substantial portion of the strains is apparently lacking the antigen that is supposed to give rise to the potentially pathogenic anti-ganglioside antibodies. Further examination of the disease etiology in these patients is mandatory.

Acknowledgments

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Supplementary Tables I to XXVII

Supplementary Table I Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB1 using a basic structure containing Kdo_2 Hep_2 Hex_5.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3060.0	3059.89
GlcN3N-GlcN-2*PP*Etn	3183.0	3182.94
GlcN3N-GlcN3N-*PP*Etn	3285.0	3285.27
GlcN3N-GlcN3N-2*PP*Etn	3408.0	3408.32

Assignments are from a comparison of the observed and calculated mass (Da) based on the proposed structures (Table 2). Average mass units were used for calculation of molecular mass values based on proposed compositions as follows: Glc/Gal, 162.14; HexNAc, 203.19; Hep, 192.17; Kdo, 220.18; GlcN, 161.16; GlcN3N 160.18; NeuAc, 291.26; phosphate, 79.98; *P*Etn, 123.05; phosphoramidate, 78.98; 3-OH C14:0 fatty acid, 226.36.

Supplementary Table II Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB2 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN	-	3108.0	3107.01
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3522.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation

Supplementary Table III Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB3 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3744.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table IV Assignment of the variants for the lipid A backbone and variable hexose (Hex) of the *O*-deacylated LOS from *C. jejuni* GB4 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_3$ $QuiNAc_1$ $phosphoramidate_1$.

Lipid A variant	Hex	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3327.6	3326.16
GlcN3N-GlcN-2*PP*Etn	-	3450.9	3449.21
GlcN3N-GlcN-*PP*Etn	+	3489.7	3488.30
GlcN3N-GlcN3N-*PP*Etn	-	3553.3	3551.54
GlcN3N-GlcN-2*PP*Etn	+	3613.3	3611.35
GlcN3N-GlcN3N-2*PP*Etn	-	3676.2	3674.59
GlcN3N-GlcN3N-*PP*Etn	+	3715.1	3713.68
GlcN3N-GlcN3N-2*PP*Etn	+	3838.3	3836.73

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table V Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB5 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN-GlcN-*PP*Etn	2713.5	2713.42
GlcN3N-GlcN	2815.5	2815.75
GlcN3N-GlcN-*PP*Etn	2938.3	2938.80
GlcN3N-GlcN-2*PP*Etn	3061.8	3061.85
GlcN3N-GlcN3N-*PP*Etn	3166.3	3164.18
GlcN3N-GlcN3N-2*PP*Etn	3286.3	3287.23

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table VI Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB11 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3230.4	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3354.1	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.8	3455.44
GlcN3N-GlcN-*PP*Etn	+	3522.7	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3580.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.9	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3748.2	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3871.1	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table VII Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB13 using a basic structure containing Kdo_2 Hep_2 Hex_6 $HexNAc_1$ $NeuAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3717.0	3716.48
GlcN3N-GlcN-2*PP*Etn	3840.0	3839.53
GlcN3N-GlcN3N-*PP*Etn	3942.0	3941.86
GlcN3N-GlcN3N-2*PP*Etn	4065.0	4064.91

See Supplemetary Table I for the average mass units used for calculation.

Supplementary Table VIII Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB14 using a basic structure containing Kdo_2 Hep_2 Hex_6 $HexNAc_1$ $NeuAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3717.0	3716.48
GlcN3N-GlcN-2*PP*Etn	3840.0	3839.53
GlcN3N-GlcN3N-*PP*Etn	3942.0	3941.86
GlcN3N-GlcN3N-2*PP*Etn	4065.0	4064.91

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table IX Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB15 using a basic structure containing Kdo_2 Hep_2 Hex_5 $HexNAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3141.0	3140.03
GlcN3N-GlcN-2*PP*Etn	3261.0	3263.08
GlcN3N-GlcN3N-*PP*Etn	3365.0	3365.41
GlcN3N-GlcN3N-2*PP*Etn	3488.5	3488.46

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table X Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB16 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$ $NeuAc_2$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3684.0	3683.46
GlcN3N-GlcN-2*PP*Etn	3805.5	3806.51
GlcN3N-GlcN3N-*PP*Etn	3908.5	3908.84
GlcN3N-GlcN3N-2*PP*Etn	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XI Assignment of the variants for the lipid A backbone and variable terminal sialic acids (NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB17 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage while terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8-linkage.

Lipid A variant	t-2,3 NeuAc	t-2,8 NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN3N-*PP*Etn	-	-	3327.0	3326.32
GlcN3N-GlcN-*PP*Etn	+	-	3393.0	3392.20
GlcN3N-GlcN-2*PP*Etn	+	-	3513.0	3515.25
GlcN3N-GlcN3N-*PP*Etn	+	-	3618.0	3617.58
GlcN3N-GlcN-*PP*Etn	+	+	3684.0	3683.46
GlcN3N-GlcN-2*PP*Etn	+	+	3807.0	3806.51
GlcN3N-GlcN3N-*PP*Etn	+	+	3909.0	3908.84
GlcN3N-GlcN3N-2*PP*Etn	+	+	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XII Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB18 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XIII Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB19 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$ $NeuAc_2$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3684.0	3683.46
GlcN3N-GlcN-2*PP*Etn	3807.0	3806.51
GlcN3N-GlcN3N-*PP*Etn	3909.0	3908.84
GlcN3N-GlcN3N-2*PP*Etn	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XIV Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB21 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3 NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3744.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XV Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB22 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XVI Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB23 using a basic structure containing Kdo_2 Hep_2 Hex_2 $HexNAc_1$ $NeuAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3066.0	3067.92
GlcN3N-GlcN-2*PP*Etn	3189.0	3190.97
GlcN3N-GlcN3N-*PP*Etn	3291.0	3293.30
GlcN3N-GlcN3N-2*PP*Etn	3414.0	3416.35

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XVII Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB24 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$ P_2.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3582.8	3585.18
GlcN3N-GlcN-2*PP*Etn	3705.8	3708.23
GlcN3N-GlcN3N-*PP*Etn	3808.2	3810.56
GlcN3N-GlcN3N-2*PP*Etn	3931.6	3933.61

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XVIII Assignment of the variants for the lipid A backbone and variable terminal sialic acids (NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB25 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage while the terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8- linkage.

Lipid A variant	t-2,3-NeuAc	t-2,8-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-2*PP*Etn	-	-	3225.0	3223.99
GlcN3N-GlcN3N-*PP*Etn	-	-	3327.0	3326.32
GlcN3N-GlcN-*PP*Etn	+	-	3393.0	3392.20
GlcN3N-GlcN3N-2*PP*Etn	-	-	3447.0	3449.37
GlcN3N-GlcN-2*PP*Etn	+	-	3513.0	3515.25
GlcN3N-GlcN3N-*PP*Etn	+	-	3618.0	3617.58
GlcN3N-GlcN-*PP*Etn	+	+	3681.0	3683.46
GlcN3N-GlcN3N-2*PP*Etn	+	-	3741.0	3740.63
GlcN3N-GlcN-2*PP*Etn	+	+	3807.0	3806.51
GlcN3N-GlcN3N-*PP*Etn	+	+	3909.0	3908.84
GlcN3N-GlcN3N-2*PP*Etn	+	+	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XIX Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB26 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3099.0	3100.94
GlcN3N-GlcN-2*PP*Etn	-	3222.0	3223.99
GlcN3N-GlcN3N-*PP*Etn	-	3327.0	3326.32
GlcN3N-GlcN-*PP*Etn	+	3393.0	3392.20
GlcN3N-GlcN3N-2*PP*Etn	-	3447.0	3449.37
GlcN3N-GlcN-2*PP*Etn	+	3513.0	3515.25
GlcN3N-GlcN3N-*PP*Etn	+	3618.0	3617.58
GlcN3N-GlcN3N-2*PP*Etn	+	3741.0	3740.63

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XX Assignment of the variants for the lipid A backbone of the *O*-deacylated LOS from *C. jejuni* GB27 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$.

Lipid A variant	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	3099.0	3100.94
GlcN3N-GlcN-2*PP*Etn	3222.0	3223.99
GlcN3N-GlcN3N-*PP*Etn	3324.0	3326.32
GlcN3N-GlcN3N-2*PP*Etn	3447.0	3449.37

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXI Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB28 using a basic structure containing Kdo_2 Hep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3231.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3453.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3519.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXII Assignment of the variants for the lipid A backbone and variable terminal sialic acid (t-2,3-NeuAc) of the *O*-deacylated LOS from *C. jejuni* GB31 using a basic structure containing Kdo_2 H

ep_2 Hex_3 $HexNAc_1$ $NeuAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage.

Lipid A variant	t-2,3-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3228.0	3230.06
GlcN3N-GlcN-2*PP*Etn	-	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	-	3456.0	3455.44
GlcN3N-GlcN-*PP*Etn	+	3522.0	3521.32
GlcN3N-GlcN3N-2*PP*Etn	-	3579.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	3747.0	3746.70
GlcN3N-GlcN3N-2*PP*Etn	+	3870.0	3869.75

See Table I for the average mass units used for calculation.

Supplementary Table XXIII Assignment of the variants for the lipid A backbone and variable terminal sialic acid (NeuAc) of the *O*-deacylated LOS from *C. jejuni* MF6 using a basic structure containing Kdo_2 Hep_2 Hex_4 $HexNAc_1$ $NeuAc_1$. The terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8- linkage.

Lipid A variant	t-2,8-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	3390.0	3392.20
GlcN3N-GlcN-2*PP*Etn	-	3513.0	3515.25
GlcN3N-GlcN3N-*PP*Etn	-	3618.0	3617.58
GlcN3N-GlcN-*PP*Etn	+	3684.0	3683.46
GlcN3N-GlcN-2*PP*Etn	+	3807.0	3806.51
GlcN3N-GlcN3N-*PP*Etn	+	3909.0	3908.84
GlcN3N-GlcN3N-2*PP*Etn	+	4032.0	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXIV Assignment of the variants for the lipid A backbone, variable terminal sialic acids (NeuAc) and HexNAc of the *O*-deacylated LOS from *C. jejuni* MF7 using a basic structure containing Kdo_2 Hep_2 Hex_3 $NeuAc_1$. The terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8-linkage.

Lipid A variant	t-2,8-NeuAc	HexNAc	Observed mass	Calculated mass
GlcN3N-GlcN-2*PP*Etn	-	+	3354.0	3353.11
GlcN3N-GlcN-2*PP*Etn	+	-	3441.0	3441.18
GlcN3N-GlcN3N-*PP*Etn	+	-	3543.0	3543.51
GlcN3N-GlcN3N-2*PP*Etn	-	+	3576.0	3578.49
GlcN3N-GlcN-2*PP*Etn	+	+	3645.0	3644.37
GlcN3N-GlcN3N-*PP*Etn	+	+	3747.0	3746.70
GlcN3N-GlcN3N-*PP*Etn	3	-	3834.0	3834.77
GlcN3N-GlcN3N-2*PP*Etn	+	+	3870.0	3869.75
GlcN3N-GlcN3N-2*PP*Etn	3	-	3957.0	3957.82
GlcN3N-GlcN3N-*PP*Etn	3	+	4038.0	4037.96

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXV Assignment of the variants for the lipid A backbone, variable terminal sialic acids (NeuAc) and HexNAc of the *O*-deacylated LOS from *C. jejuni* MF8 using a basic structure containing Kdo_2 Hep_2 Hex_3 $NeuAc_1$. The terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8-linkage.

Lipid A variant	t-2,8-NeuAc	HexNAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	-	-	3027.0	3026.87
GlcN3N-GlcN-2*PP*Etn	-	-	3150.0	3149.92
GlcN3N-GlcN-2*PP*Etn	-	+	3351.0	3353.11
GlcN3N-GlcN3N-*PP*Etn	+	-	3543.0	3543.51
GlcN3N-GlcN3N-2*PP*Etn	-	+	3579.0	3578.49
GlcN3N-GlcN3N-2*PP*Etn	+	-	3666.0	3666.56

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXVI Assignment of the variants for the lipid A backbone and variable terminal sialic acids (NeuAc) of the *O*-deacylated LOS from *C. jejuni* MF20 using a basic structure containing

Kdo_2 Hep_2 Hex_4 $HexNAc_1$. The sialic acid (t-2,3-NeuAc) linked to the terminal Gal is presumed to be linked through an α-2,3- linkage while terminal sialic acid (t-2,8-NeuAc) is presumed to be linked through an α-2,8-linkage.

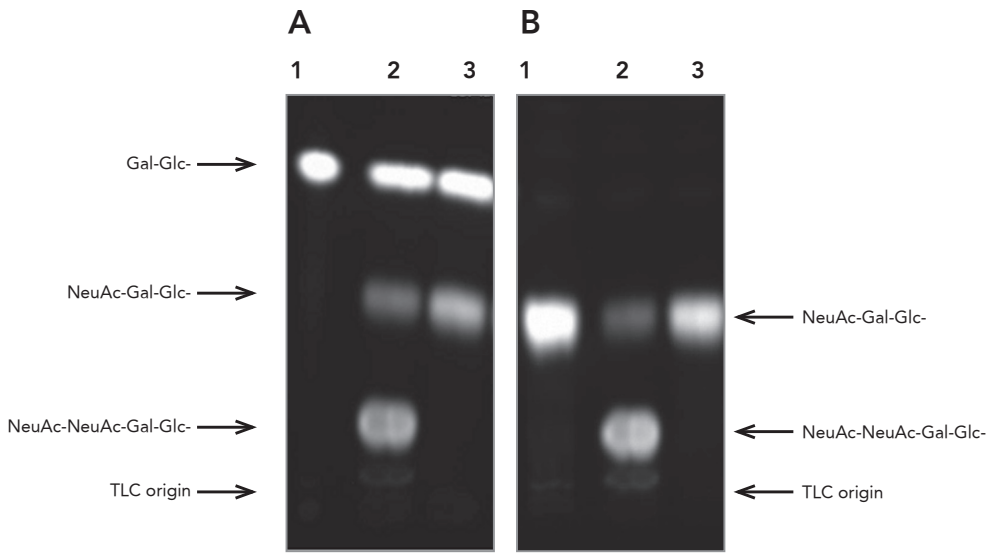
Lipid A variant	t-2,3-NeuAc	t-2,8-NeuAc	Observed mass	Calculated mass
GlcN3N-GlcN-*PP*Etn	+	-	3392.2	3392.20
GlcN3N-GlcN-2*PP*Etn	+	-	3516.3	3515.25
GlcN3N-GlcN3N-*PP*Etn	+	-	3618.9	3617.58
GlcN3N-GlcN-*PP*Etn	+	+	3684.3	3683.46
GlcN3N-GlcN3N-2*PP*Etn	+	-	3742.1	3740.63
GlcN3N-GlcN-2*PP*Etn	+	+	3807.1	3806.51
GlcN3N-GlcN3N-*PP*Etn	+	+	3910.3	3908.84
GlcN3N-GlcN3N-2*PP*Etn	+	+	4033.1	4031.89

See Supplementary Table I for the average mass units used for calculation.

Supplementary Table XXVII: Information about the DNA sequencing of the lipooligosaccharide loci from GBS- and MFS-associated strains.

Strain	GenBank Accession number	Contig size (bp)	Double-Strand coverage (%)
GB1	EF066651	1,906	98.3
GB2	DQ813306	6,047	86.1
GB3	DQ906040	6,047	99.4
GB4	AY943308	15,092	96.9
GB5	AY854153	12,403	93.0
MF6	AY422196	12,370	85.8
MF7	DQ140270	12,354	89.3
MF8	DQ102714	12,359	92.3
GB11	AY422197	24,425	98.0
GB13	EF101695	1,906	99.0
GB14	EF101696	1,906	96.3
GB15	AY423554	7,633	87.5
GB16	EF076703	6,036	93.7
GB17	EF094857	7,930	86.0
GB18	DQ868320	6,047	99.4
GB19	DQ357237	11,413	73.1
MF20	EF064287	9,357	96.3
GB21	EF076704	6,047	98.0
GB22	EF091821	6,047	97.8
GB23	EF107518	6,045	83.3
GB24	AY573819	9,295	98.3
GB25	EF064288	9,357	91.5
GB26	DQ351737	11,427	85.3
GB27	EF095404	880	1.2 [a]
GB28	DQ906041	6,047	99.1
GB31	DQ518908	11,419	96.9

[a] Only the phase-variable *cstII* gene was sequenced in GB27. The heterogeneous G-tract prevented sequencing on both strands but the sequence was confirmed by multiple same strand sequencing reactions.



Supplementary Figure 1. Demonstration that Cst-II from *Campylobacter jejuni* GB26 is mono-functional. The α-2,3-sialyltransferase activity was assayed using Galβ-1,4-Glc-FCHASE as acceptor (Panel A). The α-2,8-sialyltransferase activity was assayed using Neu5Acα-2-3-Galβ-1,4-Glc-FCHASE as acceptor (Panel B). Purified recombinant MalE-Cst-II from GB26 was incubated with either Galβ-1,4-Glc-FCHASE (Panel A, lane 3) or Neu5Acα-2-3-Galβ-1,4-Glc-FCHASE (Panel B, lane 3) and a 2-fold excess of CMP-NeuAc. Product is observed only with Galβ-1,4-Glc-FCHASE (lane 3) as acceptor. Lane 1 from panel A shows where Gal-Glc-FCHASE migrates and lane 1 from panel B shows where Neu5Acα-2-3-Galβ-1,4-Glc-FCHASE migrates. Lanes 2 show an example of a Cst-II that is bifunctional (Cst-II(Ile53Ser) from *C. jejuni* OH4384).

Appendix A:

Key to LOS outer core structures from Class A: The glycosyltransferase variants for each *C. jejuni* strain are indicated in Table 3

Step 1: Glucosyltransferase variant

Two-domain Cj1135

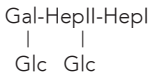
Basic inner core:

One-domain Cj1135

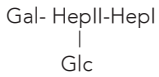
Basic inner core

Step 2: Cj1136 variant is always observed as complete product in class A strains studied in this work

Case 1: Gal extension from HepII with two glucose units on inner core

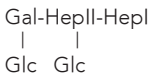


Case 2: Gal extension from HepII with one glucose unit on inner core

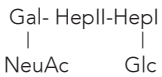


Step 3: Addition of NeuAc to the inner Gal by Cst-II when there is no Glc on HepII.

Case 1: No NeuAc on inner Gal

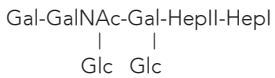


Case 2: NeuAc on inner Gal

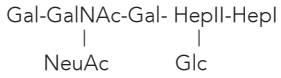


Step 4: Specificity of CgtA and CgtB

Case1: CgtA and CgtB use non-sialylated acceptors

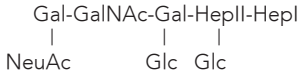


Case 2: CgtA and CgtB use sialylated acceptors

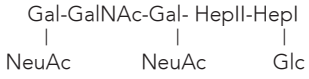


Step 5: Mono- or bi-functional Cst-II

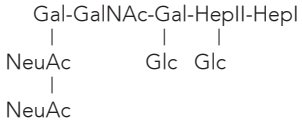
Case1.1: Mono-functional Cst-II



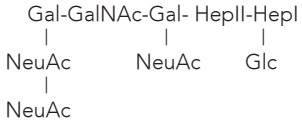
Case 1.2: Mono-functional Cst-II



Case 2.1: Bi-functional Cst-II



Case 1.2: Bi-functional Cst-II



Appendix B:

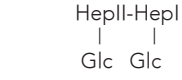
Key to LOS outer core structures from Class B:

The glycosyltransferase variants for each *C. jejuni* strain are indicated in Table 3

Step 1: Glucosyltransferase variant

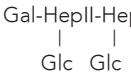
All class B strains have a two-domain Cj1135:

Basic inner core:

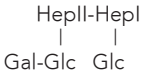


Step 2:

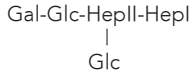
Case 1: Cj1136 encodes a full length galactosyltransferase which results in a Gal extension from HepII with two glucose units on inner core:



Case 2: Cj1136 encodes a truncated galactosyltransferase which prevent extension from HepII. An un-identified gene encodes a galactosyltransferase that uses the Glc on HepII as acceptor:

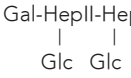


Note: equivalent to:

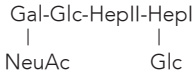


Step 3: Addition (or not) of NeuAc to the inner Gal by Cst-II:

Case 1: No NeuAc added to the Gal extension from HepII because of the presence of a Glc on HepII.

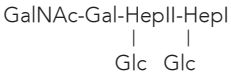


Case 2: Addition of NeuAc on Gal attached to Glc

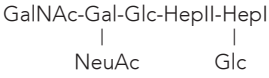


Step 4: Addition of GalNAc

Case1: CgtA uses a non-sialylated acceptor

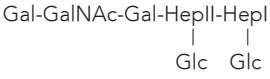


Case 2: CgtA uses a sialylated acceptor



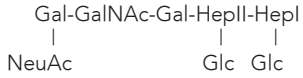
Step 5: Addition of a terminal Gal is observed only in the non-sialylated extension

Case 1:

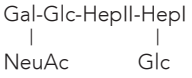


Step 6: Mono- or bi-functional Cst-II

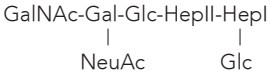
Case1.1: Mono-functional Cst-II



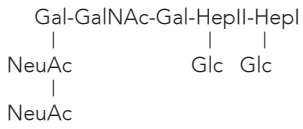
Case 1.2: Mono-functional Cst-II



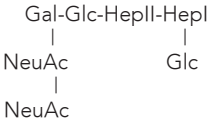
Case 1.3: Mono-functional Cst-II



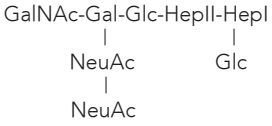
Case 2.1: Bi-functional Cst-II



Case 2.2: Bi-functional Cst-II



Case 2.3: Bi-functional Cst-II



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Chapter 7 Origin of ganglioside complex antibodies in Guillain-Barré syndrome

Under temporary embargo

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CHAPTER 8

Identification of DNA sequence variation in *Campylobacter jejuni* strains associated with the Guillain-Barré syndrome by high-throughput AFLP analysis

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Abstract

Background

Campylobacter jejuni is the predominant cause of antecedent infection in post-infectious neuropathies such as the Guillain-Barré (GBS) and Miller Fisher syndromes (MFS). GBS and MFS are probably induced by molecular mimicry between human gangliosides and bacterial lipo-oligosaccharides (LOS). This study describes a new *C. jejuni*-specific high-throughput AFLP (htAFLP) approach for detection and identification of DNA polymorphism, in general, and of putative GBS/MFS-markers, in particular.

Results

We compared 6 different isolates of the "genome strain" NCTC 11168 obtained from different laboratories. HtAFLP analysis generated approximately 3000 markers per stain, 19 of which were polymorphic. The DNA polymorphisms could not be confirmed by PCR-RFLP analysis, suggesting a baseline level of 0.6% AFLP artefacts. Comparison of NCTC 11168 with 4 GBS-associated strains revealed 23 potentially GBS-specific markers, 17 of which were identified by DNA sequencing. A collection of 27 GBS/MFS-associated and 17 enteritis control strains was analyzed with PCR-RFLP tests based on 11 of these markers. We identified 3 markers, located in the LOS biosynthesis genes cj1136, cj1138 and cj1139c, that were significantly associated with GBS ($P = 0.024$, $P = 0.047$ and $P < 0.001$, respectively). HtAFLP analysis of 13 highly clonal South African GBS/MFS-associated and enteritis control strains did not reveal GBS-specific markers.

Conclusions

This study shows that bacterial GBS markers are limited in number and located in the LOS biosynthesis genes, which corroborates the current consensus that LOS mimicry may be the prime etiologic determinant of GBS. Furthermore, our results demonstrate that htAFLP, with its high reproducibility and resolution, is an effective technique for the detection and subsequent identification of putative bacterial disease markers.

Background

The Guillain-Barré syndrome (GBS) is the most frequent form of acute immune-mediated neuropathy. The Miller Fisher syndrome (MFS) is a variant of GBS, affecting mainly the eye muscles (1). A respiratory or gastro-intestinal infection preceding the neurological symptoms is reported by nearly two-thirds of all patients (2). The most frequently identified infectious agent is *Campylobacter jejuni*, which is also the predominant cause of bacterial diarrhoea worldwide (3, 4). The neuropathy is probably induced by molecular mimicry between gangliosides in nerve tissue and lipo-oligosaccharides (LOS) on the *Campylobacter* cell surface (5). This structural resemblance leads to a cross-reactive immune response causing neurological damage. Biochemical and serological studies have revealed that many *C. jejuni* strains express ganglioside-like structures in their LOS (6). However, not all strains expressing ganglioside mimics induce GBS. It is estimated that only 1 in every 1000-3000 *C. jejuni* infections is followed by GBS (7, 8), which suggests that additional bacterial determinants and / or host-related factors are important as well.

Many researchers have studied collections of GBS-associated and control "enteritis-only" strains in search of GBS-specific microbial features. Several reports describe an overrepresentation of specific Penner (heat stable, HS) serotypes among GBS-associated strains from certain geographical areas (9, 10). The HS:19 and HS:41 serotypes are the predominant serotypes preceding GBS in Japan and South Africa, respectively (9, 10). Because HS:19 and HS:41 strains represent a clonal population (11, 12), the observed overrepresentation of these serotypes does not imply that the determinant of the Penner serotyping system, the capsular polysaccharide, is involved in the pathogenesis of GBS (13). In addition, this phenomenon is not seen in other regions, where GBS-associated strains are genetically heterogeneous (14). Various molecular typing techniques have been used in search of GBS-specific features in *C. jejuni*, such as *flaA*-PCR-restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) analysis, ribotyping, amplified fragment length polymorphism (AFLP) analysis and multi locus sequence typing (MLST), but none of these have identified GBS-specific markers (14-17). Very recently, Leonard et al. also failed to detect GBS-specific features by the use of an open reading frame (ORF)-specific *C. jejuni* DNA microarray (18). However, this array was based on the genome sequence of strain NCTC 11168 and ORFs that are not present in this strain will not be detected. In addition, possible GBS-factors other than those relating to presence or absence of certain genes will not be detected using this approach. Based on the molecular mimicry hypothesis, other researchers focused on genes involved in LOS biosynthesis and found significant associations with GBS (19-22). However, these associations are not absolute and the question remains whether other GBS-specific microbial factors, either LOS-related or not, may exist.

Comparative genomics technology facilitates genetic marker identification but not all methods may be equally suited for high-throughput marker searches. Molecular typing techniques for *Campylobacter* strains differ in sensitivity and the overall coverage of genome regions screened. For the detection of specific disease markers it is desirable to use a technique that screens diversity in the overall genome with a very high resolution. MLST and *flaA* PCR-RFLP analyze restricted parts of the genome and are not suitable for the detection of additional GBS-markers (23, 24). PFGE is based on digestion of genomic DNA with a rare cutting restriction enzyme and only large insertions or deletions and mutations in the restriction sites will be detected (14). PFGE patterns normally display between 10 and 20 fragments, which covers 120 nucleotides when a six nucleotide restriction enzyme recognition sequence is involved. AFLP analysis is considerably more sensitive than PFGE for the detection of DNA sequence polymorphism. In a conventional AFLP analysis, the use of two restriction enzymes and a primer pair with 1 or 2 selective nucleotides leads to a DNA fingerprint pattern consisting of approximately 50-80 fragments per strain. This approach physically covers in the order of 600-1000 nucleotides of the total genome for sequence polymorphism (25). Even the use of two restriction enzymes in PFGE will not make up for the difference observed under a single AFLP reaction. As indicated above, DNA microarrays cover the full genome but will only detect differences in the presence of known genes. Recently, we described a new high throughput AFLP (htAFLP) approach for the identification of DNA polymorphism in *Mycobacterium tuberculosis* (26). This method has the capacity to detect mutations in more than 30,000 nucleotides scattered throughout the genome, depending on the number of restriction enzymes and primer pairs used. The choice of primers and restriction enzymes is crucial and selection of these requires close attention. A wrong choice may lead to crowding of amplified fragments, caused by limited resolution of the gelsystem. Correct, computer-mediated comparison of AFLP fingerprints may then be compromised. However, especially the enhanced resolution makes htAFLP an excellent candidate technique for the detection of potential disease-associated markers.

The main objective of the current study was to search an elaborate collection of *C. jejuni* stains isolated from GBS patients for genetic markers associated with bacterial neuropathogenicity. To this aim, we developed htAFLP for *C. jejuni*. We analyzed six isolates of strain NCTC 11168, the "genome" strain, obtained from different laboratories, with the aim to detect base-level polymorphism introduced by sub-culturing or storage. In search of potential GBS-specific markers, we compared the NCTC 11168 AFLP patterns with those of four GBS-associated strains. In addition, we analyzed a collection of highly clonal GBS-associated and control strains from South Africa. Potential GBS-specific htAFLP markers were further identified by DNA sequencing and PCR-RFLP tests were developed. These PCR-RFLP tests were used to screen a larger collection of GBS-associated and control strains for confirmation of the potential GBS markers.

Results

Detection and identification of DNA sequence polymorphism in NCTC 11168 strains of diverse origin

HtAFLP analysis of *C. jejuni* was performed with one enzyme combination. Genomic DNA was digested with MboI and DdeI and the restricted DNA was amplified by using all 64 possible combinations of +1/+2 selective primer pairs. This resulted in the generation of approximately 3000 fragments per strain. The average fragment size was 243 basepairs (bp), ranging from 46 to 613 bp. Comparative htAFLP analysis of the six NCTC 11168 isolates revealed 19 polymorphic fragments. After excision from the gel, these fragments were amplified and the DNA sequences were determined. BLAST analysis of the DNA sequences resulted in the identification of 13/19 polymorphisms, which were spread throughout the genome (Table 1). For the other polymorphic fragments, repeated amplification and sequencing failed to generate DNA sequences of sufficient quality for BLAST analysis.

Validation of NCTC 11168 polymorphism with a PCR-RFLP approach

To verify whether the htAFLP-polymorphic fragments represent true DNA sequence polymorphism, we analyzed the six NCTC 11168 isolates by PCR-RFLP analysis. An AFLP polymorphism that is based on mutations in the restriction site will result in a polymorphic RFLP pattern, whereas insertions or deletions in the AFLP fragment will result in size differences between the PCR products. Based on the BLAST hit sequences (Table 1), PCR tests for amplifying twelve marker fragments and their flanking regions were developed. Fragments of correct size were produced with all primer sets and for all isolates (results not shown). Next, PCR products of the six NCTC 11168 strains were digested in separate reactions with the AFLP restriction enzymes (results not shown). None of the digests showed polymorphic RFLP patterns. Thus, restriction site polymorphism or insertions/deletions could not be confirmed as cause of the observed AFLP polymorphisms. Nine out of twelve (75%) digests with *DdeI* and six of twelve (50%) digests with *MboI* resulted in RFLP patterns as expected based on the NCTC 11168 DNA sequence. An AFLP polymorphism can also be the result of a mutation in the nucleotides complementary to the selective primer nucleotides. Because all 64 possible combinations of the +1/+2 primer pairs were used in this htAFLP, such a mutation would be expected to result in an additional polymorphism, with the same fragment length and localization on the genome, in the AFLP pattern generated with a different primer pair. We did not detect such complementary polymorphisms in the NCTC 11168 comparison. In conclusion, the polymorphic AFLP bands observed in the NCTC 11168 comparison, representing approximately 0.6% of all bands, probably represent the low "background noise" of the htAFLP technique.

Comparison of NCTC 11168 with GBS-associated strains for the detection and identification of potential markers for GBS

For the detection of putative markers for the Guillain-Barré syndrome, we compared the NCTC 11168 isolates with strain GB11, which was isolated from a GBS patient. Strain NCTC

Table 1. Polymorphic htAFLP markers for NCTC11168 strains of diverse origin

	NCTC11168 strains[b]							11168 hit sequence[d]		
marker[a]	1	2	3	4	5	6	length[c]	begin	end	gene and function
1	+	+	+	+	-	+	469	28753	28317	Cj0023, purB, probable adenylosuccinate lyase
2	-	-	+	-	-	+	252	67665	67858	Cj0046, probable transmembrane transport protein pseudogene
3	-	-	+	-	-	-	110	122004	122083	Cj0117, pfs, probable 5'-methylthioadenosine\S-adenosylhomocysteine nucleosidase
4	-	-	+	+	+	+	118	153984	154075	Cj0150c, probable aminotransferase
5	-	-	+	+	+	+	86	212438	212383	Cj0227, argD, probable acetylornithine aminotransferase
6	+	+	+	+	+	-	420	573124	572971	Cj0612c, cft, probable ferritin; Cj0613, pstS, possible periplasmic phosphate binding protein
7	-	-	+	+	+	+	49	589048	589393	Cj0629, possible lipoprotein; Highly similar to Cj1678; Cj1678, possible lipoprotein
8	-	-	+	+	+	+	382	788562	788468	Cj0840c, fbp, probable fructose-1,6-bisphosphatase
9	-	-	+	-	-	-	463	1047891	1048322	Cj1116c, ftsH, probable membrane bound zinc metallopeptidase
10	-	-	-	+	+	-	228	1227296	1227125	Cj1295, unknown
11	+	-	-	+	-	-	226	1227296	1227117	Cj1295, unknown
12	+	+	-	+	+	+	191	1270291	1270131	Cj1339c, flaA, flagellin A
13	-	-	+	-	+	+	126	1510286	1510381	Cj1580c, probable peptide ABC-transport system ATP-binding protein

[a]The individual markers have been given a numerical code. [b]The strains are numbered according to Additional file 1. The plusses and minuses indicate the presence and absence, respectively, of the AFLP fragments. [c]The fragment lengths, based on fragment position in the AFLP gels. [d]The begin and the end positions in the NCTC 11168 genome are given for all BLAST hits, as well as the corresponding genes and their function.

11168 was originally isolated from a patient with gastroenteritis without neurological symptoms. It had previously been shown that GB11 and NCTC 11168 are genetically closely related (14, 15, 27). Because of this relatedness, these strains are very suitable for the detection of potential GBS-specific markers. HtAFLP analysis of NCTC 11168 and GB11 generated 241 putative GBS markers. Overall, 156 of 241 markers could be successfully identified with DNA sequencing and BLAST analysis. A proportion of the marker fragments that were excised from the gel could not be reliably reamplified and were excluded from the analysis. Although BLAST searches were conducted against all DNA sequences in the Pubmed database, the most significant homology for all AFLP DNA sequences was with *C. jejuni* DNA sequences. To further reduce this excessive number of putative GBS markers, we analyzed three additional GBS-associated strains, not related to the NCTC 11168 and GB11 strains (Cura7, Cura276 and 260.94; See Additional file 1). This reduced the number of successfully sequenced putative GBS markers to 17 (Table 2). Three of these markers were located in the LOS biosynthesis gene locus. Other genes encoded a putative periplasmic protein and proteins involved in signal transduction, metabolism, transport, binding, amino acid biosynthesis, fatty acid biosynthesis and DNA replication. Three genes were of unknown function. Markers 5 and 6 displayed distinct restriction site polymorphism concordant with the AFLP polymorphism. These markers contained largely overlapping DNA sequences and showed a complementary pattern of presence and absence in the GBS-associated and control strains (Table 2). Comparison of the DNA sequences revealed that these markers were based on one DNA polymorphism: the presence of an additional restriction site in the GBS strains due to a point mutation (Figure 1).

Screening of a large strain collection for potential GBS markers by PCR-RFLP analysis

After htAFLP analysis of five strains to identify potential GBS markers, we developed PCR-RFLP tests to screen a large collection of 27 GBS/MFS-associated and 17 control enteritis strains for the presence of these markers (for a survey of these strains see Additional file 1). The strains used in the htAFLP analysis were also included in the PCR-RFLP analysis. One randomly selected NCTC 11168 isolate was included. Based on the BLAST hit sequences (Table 2), PCR tests for amplifying 11/17 marker fragments and their flanking regions were developed (See Additional file 2). Because markers 5 and 6 represented the same DNA polymorphism, they were included in one PCR test. Fragments of correct, expected size were produced with all primer sets. For 5/11 markers a PCR product was absent in a variable proportion of strains (Table 3), probably due to primer site sequence heterogeneity. For example, we have observed previously that gene *cj1136*, part of the LOS biosynthesis gene locus and containing marker 7, shows a large degree of DNA sequence heterogeneity between strains (P. Godschalk, unpublished results). This leads to primer mismatches and absence of PCR products in a proportion of strains. In 2/10 PCR tests (markers 7 and 8), the htAFLP GBS-associated strains could be distinguished from strain NCTC 11168 through the pattern of presence and absence of PCR products. PCR products for marker 7 were absent in the GBS-associated strains used in the htAFLP and present in NCTC 11168, which seemed to be in contrast with the observation that the original AFLP

Table 2. Putative GBS-markers detected by htAFLP

	strains[b]						11168 hit sequence[d]		
marker[a]	11168	GB11	cura 7	cura 276	O:41	length[c]	begin	end	gene and function
1	-	+	+	+	+	112	324441	324363	Cj0355c, probable two-component regulator
2	+	-	-	-	-	265	400499	400264	Cj0432c, murD, probable UDP-N-acetylmuramoylalanine--D-glutamate ligase
3	-	+	+	+	+	115	575807	575893	Cj0615, pstA, probable phosphate transport system permease protein
4	-	+	+	+	+	152	576061	576182	Cj0615, pstA, probable phosphate transport system permease protein
5	-	+	+	+	+	198	904760	904594	Cj0967, possible periplasmic protein
6	+	-	-	-	-	253	904817	904596	Cj0967, possible periplasmic protein
7	-	+	+	+	+	241	1070741	1070662	Cj1136, probable galactosyltransferase
8	+	-	-	-	-	138	1073190	1073082	Cj1138, probable galactosyltransferase
9	-	+	+	+	+	97	1074261	1074320	Cj1139c, probable galactosyltransferase
10	+	-	-	-	-	169	1090751	1090612	Cj1157, dnaX, probable DNA polymerase III subunit gamma
11	+	-	-	-	-	89	1092245	1092185	Cj1161c, probable cation-transporting ATPase
12	+	-	-	-	-	181	1236007	1236080	Cj1306c, unknown
13	+	-	-	-	-	137	1236406	1236298	Cj1306c, unknown, similar to Cj1310c,Cj1305c, Cj1342c, Cj0617/0618
14	+	-	-	-	-	438	1264182	1264586	Cj1336, unknown, identical to Cj1318 (except the C-term)
15	+	-	-	-	-	163	1328311	1328184	Cj1394, probable fumarate lyase; Cj1393, metC', probable cystathionine beta-lyase
16	+	-	-	-	-	203	1334951	1334779	Cj1400c, fabI, probable enoyl-[acyl-carrier-protein] reductase [NADH]
17	+	-	-	-	-	102			Cj1101, probable ATP-dependent DNA helicase

[a]The individual markers have been given a numerical code. [b]The plusses and minuses indicate the presence and absence, respectively, of the AFLP fragments. [c]The estimated fragment lengths on the AFLP gels are given. [d]The begin and the end positions in the NCTC 11168 genome are given for all BLAST hits, as well as the corresponding genes and their function. Note that markers 5 and 6 are largely overlapping and have a complementary pattern of presence and absence (see also Figure 1).

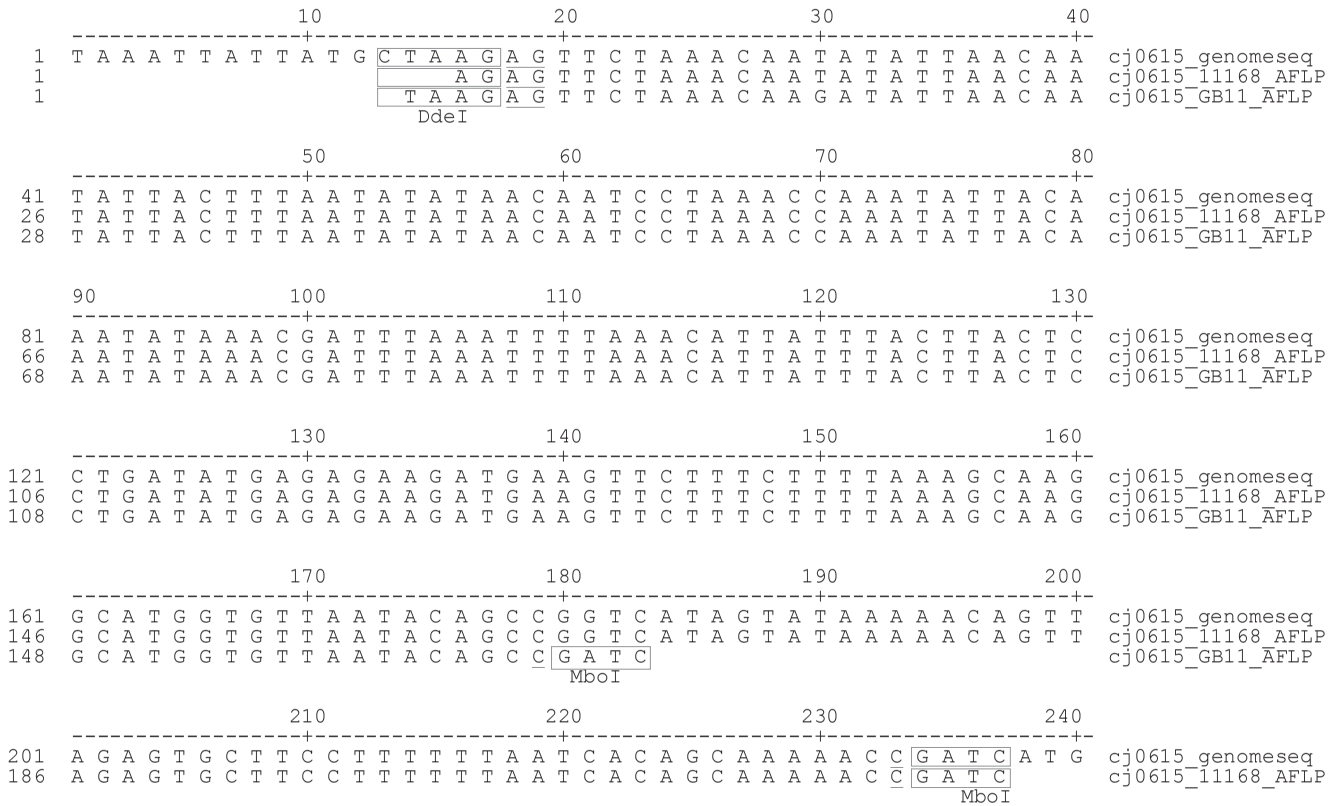

Figure 1. The basis of the AFLP polymorphism in the complementary markers 5 and 6. Marker 5 represents a fragment with a length of 198 bp that was present in the ht AFLP GBS strains, whereas marker 6, a fragment of 253 bp, was only present in the NCTC 11168 isolates. DNA sequence analysis of the GB11 and NCTC 11168 AFLP fragments and subsequent BLAST searches showed that both markers were located in gene cj0615, encoding a possible periplasmic protein. Furthermore, DNA sequence analysis revealed that a point mutation in the GBS strains had resulted in an additional MboI restriction site, resulting the amplification of a shorter AFLP fragment in the GBS strains. Because the selective nucleotide flanking the MboI restriction site was identical for the GB11 and NCTC 11168 fragment, the GBS fragments were amplified with the same primerpair as the NCTC 11168 fragments. DNA sequences of markers 5 (cj0615_GB11_AFLP) and 6 (cj0615_NCTC 11168_AFLP) are given, as well as the NCTC 11168 genome sequence of the same area (cj0615_genomeseq). The AFLP restriction sites are indicated with boxes, the selective nucleotides are underscored and the point mutation is indicated in bold.

Table 3. Results of the PCR-RFLP analysis for 11 potential GBS markers identified by htAFLP analysis.

		marker nr (genomic localization)[b]									
strain[a]	disease	2 (cj0432)	3 (cj0615)	5,6 (cj0967)	7 (cj1136)	8 (cj1138)	9 (cj1139c)	11 (cj1161c)	12 (cj1306)	13 (cj1306)	14 (cj1336)
GB1	GBS	1	1	1	1	1	0	1	2	2	1
GB2	GBS	2	2	2	-	-	1	2	4	3	-
GB3	GBS	2	2	2	-	-	1	2	3	3	-
GB4	GBS	1	2	2	-	-	2	2	1	1	-
GB5	GBS	1	2	1	-	-	3	0	5	4	-
MF6	MFS	2	2	2	-	-	4	2	6	0	-
MF7	MFS	1	2	1	-	-	3	1	5	4	-
MF8	MFS	1	2	2	-	-	0	2	6	1	-
GB11	GBS	1	2	2	-	-	4	2	7	5	-
GB13	GBS	1	2	1	1	0	0	2	5	1	-
GB15	GBS	1	1	3	1	-	0	2	5	4	2
GB16	GBS	1	1	1	-	-	3	2	1	1	-
GB17	GBS	1	2	1	-	0	0	1	5	4	-
GB18	GBS	2	2	2	-	-	4	2	4	6	-
GB19	GBS	1	1	1	-	-	5	2	3	7	-
MF20	MFS	1	2	3	-	-	5	1	5	8	-
GB21	GBS	2	2	2	-	-	4	2	0	5	3
GB23	GBS	1	2	1	-	-	4	0	1	1	4
GB24	GBS	2	1	2	-	-	-	1	1	4	-
GB25	GBS	1	2	-	-	-	3	1	5	8	-
GB26	GBS	1	1	1	-	-	3	1	5	1	-
GB29	GBS	1	2	1	1	-	-	1	10	10	3
GB30	GBS	3	1	3	1	-	3	1	1	1	5
cura 7	GBS	2	2	2	-	-	4	2	8	5	3
cura 69	GBS	2	2	2	-	-	4	2	3	9	-
cura 276	GBS	2	2	2	-	-	4	2	8	5	3
260.94	GBS	2	2	2	-	-	4	4	9	5	-
E97-0737	enteritis	1	2	1	1	nd	0	1	2	1	-
E97-0747	enteritis	1	1	1	1	nd	0	2	5	10	5
E97-0796	enteritis	1	2	1	1	-	0	1	5	4	6
E97-0873	enteritis	2	3	2	-	-	0	2	5	nd	4
E97-0903	enteritis	1	2	1	1	-	2	1	11	8	-
E97-0921	enteritis	1	2	3	-	-	6	0	11	6	-
E97-0974	enteritis	1	2	1	1	1	0	nd	5	1	4
E97-0980	enteritis	1	1	1	-	-	5	1	5	1	-
E97-0998	enteritis	2	2	2	-	-	7	2	11	nd	-
E97-1013	enteritis	1	1	3	-	-	5	1	5	10	-
E98-623	enteritis	2	2	2	-	-	2	2	5	nd	4
E98-624	enteritis	2	2	2	1	-	2	2	2	nd	7
E98-682	enteritis	1	1	1	1	1	0	1	2	10	-
E98-706	enteritis	1	1	3	-	-	0	2	1	4	-
E98-1033	enteritis	1	2	3	-	-	1	1	1	1	-
E98-1087	enteritis	1	2	1	1	1	0	1	1	4	-
NCTC 11168-1	enteritis	1	1	1	1	1	0	1	5	1	4

[a]Strains that were used in the htAFLP are indicated in bold. [b]The marker numbers correspond with the marker numbers displayed in Table 3. For each marker, the different RFLP types are indicated with numbers. 0 = single band (no restriction), - = no PCR product, nd = not determined.

fragment of marker 7 was present in the GBS strains and absent in NCTC 11168. However, this apparent inconsistency can be explained by the fact that the primer sequences of the PCR test were based on the NCTC 11168 DNA sequence. For marker 7, a PCR product was seen significantly more frequently in control enteritis strains (5/27 (18.5%) GBS/MFS strains versus 9/17 (52.9%) control enteritis strains, $P = 0.024$). Next, we subjected the PCR products to a combined digestion with the AFLP restriction enzymes (Table 3). In 4/10 PCR tests (markers 3, 5/6, 9 and 13), the RFLP types were concordant with the AFLP analysis i.e. the htAFLP GBS-associated strains shared the same RFLP type whereas NCTC 11168 displayed a different RFLP type. In 3/10 PCR tests (markers 11, 12 and 14), the htAFLP GBS-associated strains did not have identical RFLP types (and for marker 14 there was no PCR product in two GBS strains), but these RFLP types were also different from that of NCTC 11168. This is not necessarily in contrast with the htAFLP results, because different RFLP types among the htAFLP GBS-associated strains may be due to heterogeneity in the flanking regions of the AFLP fragment. For marker 2, the RFLP types of the htAFLP strains were not concordant with the htAFLP polymorphism: the NCTC 11168 and GB11 RFLP types were the same. RFLP types 3 and 4 of marker 9, located in gene cj1139c, were only detected in GBS/MFS-associated strains (RFLP type 3 or 4 present in 15/27 (55.6 %) GBS/MFS-associated strains versus 0/17 (0%) enteritis strains, $P < 0.0001$). Although a PCR product for marker 8, located in gene cj1138, was absent in the majority of strains, RFLP type 1 was more frequently found in enteritis strains (5/15 enteritis strains versus 1/27 GBS-associated strains, $P = 0.047$).

Analysis of South-African GBS-associated and control HS:41 strains by htAFLP

In South Africa, serotype HS:41 is over-represented among GBS-associated strains (10). Previous studies have shown that HS:41 strains, both GBS-associated and controls, are highly clonal (12). As expected, htAFLP of six GBS-associated, one MFS-associated and six control HS:41 strains generated very homogeneous banding patterns (results not shown). A total of forty-five AFLP polymorphisms were detected, but there were no GBS-specific markers. Interestingly, 28 AFLP polymorphisms displayed a similar pattern: fragments were present in the MFS-associated strain and two or three control enteritis strains but absent in the other strains (Figure 2). These fragments were excised and DNA sequences were determined. BLAST analysis of five DNA sequences revealed homologies with various bacterial plasmidal DNA sequences (results not shown).

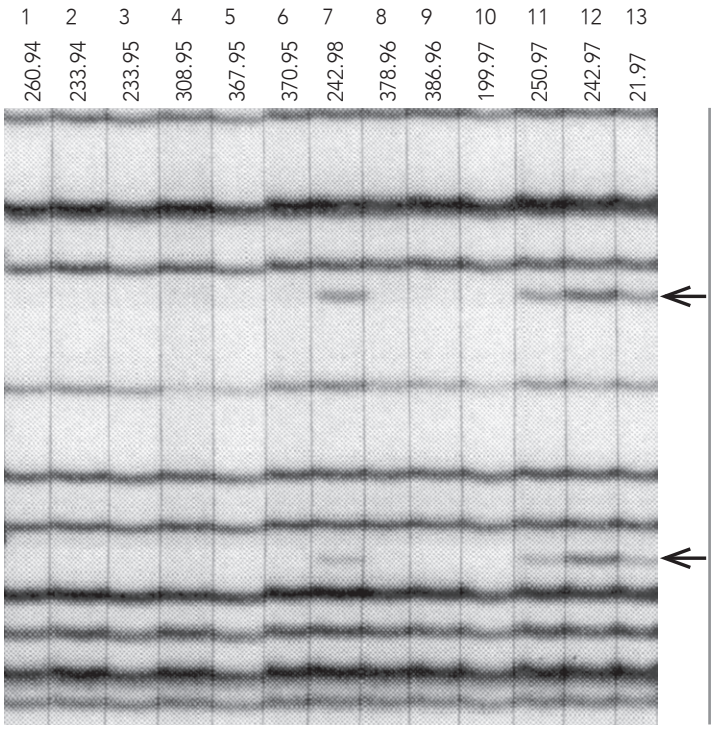

Figure 2. Detail of the htAFLP pattern of the HS:41 strains. A fragment of the banding patterns of the South African HS:41 strains is shown. Lane 1-6 GBS-associated strains, lane 7 MFS-associated strain, lane 8-13 enteritis strains. Polymorphic bands representing plasmidal DNA sequences are indicated with arrows.

Discussion

This study describes a high-throughput AFLP approach for the detection and identification of DNA polymorphism and putative GBS-markers in *C. jejuni*. Previously, we showed that htAFLP is an excellent tool for assessing the population structure and the expansion of pathogenic clones in *Staphylococcus aureus* and for identification of genetic polymorphism in the clonal micro-organism *Mycobacterium tuberculosis* (26, 28). The optimal enzyme and selective primer pair combinations are determined by *in silico* calculations using the whole genome DNA sequence of the target microorganism. The optimal number of AFLP fragments to be generated depends on the aim of the study. For the detection and identification of potential disease markers, such as GBS-specific markers in *C. jejuni* in the current study, it is desirable to screen the genome with high resolution. For this, the generation of a large number of AFLP fragments per strain is needed. Such high resolution AFLP approach limits the number of strains that can be analyzed, but this can be overcome by the subsequent analysis of a large number of strains by PCR-RFLP

tests, translated from the potential markers as detected by the preceding htAFLP analysis. It is, of course, possible that a disease-specific marker is not detected by htAFLP because this approach does not result in 100% genome coverage, which can only be reached with whole genome sequencing. For *C. jejuni*, one enzyme combination (*Mbo*I and *Dde*I) and 64 different +1/+2 selective primer pair combinations physically covered approximately 30,000 nucleotides per strain, which represents approximately 2% of the genome.

To find out whether subculturing or storage of *C. jejuni* strains leads to the emergence of DNA polymorphism, we compared 6 isolates of the "genome" strain NCTC 11168 obtained from different laboratories worldwide by htAFLP analysis. The observed AFLP polymorphisms, approximately 0.6% of the fragments per strain, could not be confirmed by PCR-RFLP analysis. This indicates that the AFLP polymorphisms probably represent the low background noise of the htAFLP technique and that true DNA polymorphism could not be detected in the six NCTC 11168 isolates. The observed background noise equals a reproducibility of approximately 99.6%, which is still very high when compared to other genotyping techniques (29).

Recently, two groups described differences in virulence properties, i.e. the ability to colonise chickens, between different NCTC 11168 isolates that were not included in the current study (30, 31). Full transcriptional profiling revealed expression differences for several gene families in the NCTC 11168 strains. HtAFLP analysis of the two NCTC 11168 isolates that were studied by Carrillo et al. (30, 31) failed to identify polymorphisms responsible for the difference in virulence properties (P. Godschalk and C. Szymanski, unpublished data). It has to be emphasized that by htAFLP still only a random proportion of the genome is screened. DNA sequence variation (such as single-nucleotide polymorphisms, SNPs) leading to biological differences may therefore not be detected.

In search of GBS-specific markers, we first compared the NCTC 11168 patterns with the AFLP patterns of the genetically related GBS-associated strain GB11. However, although NCTC 11168 was originally isolated from the faeces of a patient with gastroenteritis, we cannot exclude that NCTC 11168 can induce GBS if a patient with the right host susceptibility factors becomes infected with this strain. The only substantial but probably very important difference between NCTC 11168 and GB11 that has been found so far, is that the LOS biosynthesis gene locus strongly diverges between these strains, probably as result of a horizontal exchange event (32). Comparison of NCTC 11168 with GB11 led to the detection of more than two hundred possible GBS markers, which was substantially higher than the expected background noise of 0.6%, underscoring the phylogenetic relevance of the polymorphisms. The number of possible GBS markers was reduced to 23 after adding three additional GBS-associated strains. For 17 markers, the location on the genome could be identified after DNA sequence analysis. A relatively large proportion of potential GBS markers (3/17;18%) was located in the LOS biosynthesis gene locus, whereas this locus only comprises 1% of the *C. jejuni* genome (1.64 Mbp). Although this may represent a true pathogenic association with GBS, it is also possible that htAFLP preferentially

picked up the LOS locus because it is a highly polymorphic region. However, analysis of a larger *C. jejuni* strain collection by PCR-RFLP analysis showed that the three LOS-specific markers were indeed associated with GBS (marker 7, $P = 0.024$; marker 8, $P = 0.047$; marker 9, $P < 0.001$). These findings are concordant with the proposed pathogenic mechanism of GBS and with previous reports that certain genes involved in LOS biosynthesis or specific nucleotide sequences within these genes occur more frequently in GBS-associated *C. jejuni* strains (19-22).

There are several possible explanations for the fact that we did not find molecular markers that are 100% specific for the Guillain-Barré syndrome. First, it is possible that truly GBS-specific features do not exist in *C. jejuni*. There is a broad variability in the severity and spectrum of clinical symptoms in GBS patients (33). Different ganglioside mimicking structures and anti-ganglioside antibody specificities have been associated with certain clinical presentations (34-36), and therefore, *C. jejuni* markers may be associated with a subset of various disease entities. Because of this heterogeneity and the presumed importance of host-related factors, the existence of features in *C. jejuni* that are specific for GBS may be questionable. Second, a certain combination of multiple *C. jejuni* genes may be required for the induction of GBS. Detection of such combinations of markers ("polygenic markers") is extremely labour-intensive and cannot be achieved with the current approach. Finally, it is possible that htAFLP failed to detect GBS-specific markers because htAFLP does not accomplish 100% genome coverage.

One of the three additional GBS-associated strains mentioned above was from a collection of South-African HS:41 strains. In South Africa, the HS:41 serotype is over-represented among GBS-associated strains (10). A certain feature of these strains may be responsible for their capacity to trigger GBS. HS:41 strains were found to be indistinguishable by previous genotyping studies, indicating that HS:41 strains form a genetically stable clone (12). It is important to note that the enteritis-only HS:41 strains may have the same GBS-inducing capacity as the GBS-associated strains, because host-related factors are also crucial for developing GBS. We analyzed both GBS-associated and control enteritis-only HS:41 strains, as well as an MFS-associated isolate by htAFLP. Although we did not detect GBS-specific bands, we found that the MFS-associated isolate and half of the enteritis-only strains contained several additional fragments that appeared to be linked. DNA sequences of these fragments showed homologies with plasmidal DNA sequences, indicating that a subset of the HS:41 strains contained a plasmid. To our knowledge, the South African HS:41 strains we used in this study have never been analyzed for the presence of plasmids. Whether the presence of a plasmid is of importance for the virulence or neuropathogenic potential of HS:41 strains currently remains unknown, but seems unlikely based on the distribution of plasmidal DNA in the tested strains.

Conclusions

Previous searches for *C. jejuni* markers for GBS-invoking potential were unsuccessful when performed with general genotyping techniques. Some studies that focussed at specific loci or sometimes even specific genes found potential, though not absolute, GBS markers within the LOS biosynthesis genes (19-22). We have used a method, htAFLP, that detects sequence polymorphisms in a wide, non-gene dependent scale. Theoretically approximately 2% of the total genome is covered by this approach. However, we still conclude that bacterial GBS markers are not absolute, limited in number and located in the LOS biosynthesis gene locus. This corroborates the current consensus that LOS mimicry with human gangliosides may be the prime etiologic determinant of GBS. In addition to bacterial factors, host-related factors probably play an important role in the pathogenesis of GBS as well.

Furthermore, our results demonstrate that htAFLP, with its high reproducibility and resolution, is an adequate technique for the detection and subsequent identification of putative disease and epidemiological markers. Analysis of a limited number of strains in great detail by htAFLP and subsequent screening of a large collection of strains with simple PCR-RFLP tests combines high sensitivity with the possibility to screen large groups of strains. This allows for the identification of regions of genomic instability or variability. Finally, htAFLP does not require complete genome sequences and it is not influenced by the presence of sequences not present in the genome strain(s). As such, htAFLP is the second best option, after complete sequencing of the genome of multiple strains, for the unbiased detection of genome polymorphisms associated with pathogenicity or other features of bacterial isolates from diverse clinical and environmental origin.

Methods

Bacterial strains, culture conditions and DNA isolation

The *C. jejuni* strains used for htAFLP analysis are described in Additional file 1. We collected 6 isolates of the "genome" strain NCTC 11168 strains from different labs worldwide (37). For the detection of potential GBS markers, we included four *C. jejuni* strains isolated from the diarrhoeal stools of GBS patients from different geographical areas (The Netherlands, Curaçao, South Africa). In addition, we analyzed a collection of 6 GBS-associated, 1 MFS-associated and 6 enteritis-only HS:41 strains isolated from South African patients (12). After identification of potential GBS markers by htAFLP analysis of these strains, we screened a larger collection of 27 GBS/MFS-associated and 17 control strains isolated from enteritis patients with PCR-RFLP tests for the presence of these markers (See Additional file 1). *C. jejuni* strains were cultured for 24-48 hours on blood agar plates in a micro-aerobic atmosphere at 37°C. DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

High-throughput AFLP

AFLP analysis was performed essentially as described by Vos et al. (38). The optimal enzyme and primer combinations for *C. jejuni* were determined using the predictive software package REcomb (39). Digestion with *Mbo*I and *Dde*I (Boehringer-Mannheim, Mannheim, Germany) was combined with the ligation of a specific linker oligonucleotide pair (*Mbo*I: 5'-CTCGTAGACT-GCGTACC-3' and 5'-GATCGGTACGCAGTCTAC-3'; *Dde*I: 5'-GACGATGAGTCCTGAG-3' and 5'-TNACTCAGGACTCAT-3'). Subsequently, a non-selective pre-amplification was performed using the *Mbo*I primer (5'-GTAGACTGCGTACCGATC-3') and *Dde*I primer (5'-GACGATGAGTC-CTGAGTNAG-3'). The selective amplifications were performed using different linker-specific primer combinations. The ^{33}P-labeled *Mbo*I primer was extended with a single nucleotide (+1), whereas the *Dde*I primer was equipped with a 3' terminal dinucleotide (+2). These nucleotides probe sequence variation beyond that present in the restriction site itself. All 64 possible extension combinations were used. Radioactive labelling was used to enable isolation of DNA fragments from gels for post-AFLP sequencing analysis. Amplified material was analyzed on 50x30 cm slabgels and the amplimers were visualized using phosphor-imaging. Post-AFLP, gels were fixed, dried and stored at ambient temperature.

Marker selection and identification

Marker bands were scored using the automated interpretation software package AFLP QuantarPro (Keygene N.V., Wageningen, The Netherlands), resulting in a binary table scoring marker fragment absence (0) or presence (1). Polymorphic marker fragments were validated by visual inspection of the autoradiographs. Bands differing in signal intensity were not considered to be polymorphic. A potential marker for GBS was defined as an AFLP polymorphism that discerns the GBS-associated strains from the NCTC 11168 isolates. Potential GBS marker fragments can either be present or absent in GBS-associated strains as compared to NCTC 11168.

Relevant fragments were excised from the gels and re-amplified using their matching AFLP consensus primer set without restriction site-specific +1 and +2 extension sequences attached. The amplimers were subjected to DNA sequencing using a 96-well capillary sequencing machine (MegaBACE; Amersham). For fragment identification, the DNA sequences were subjected to BLASTn and BLASTx searches through the NCBI website (40). BLAST results enable genomic localization and gene annotation for the polymorphic marker fragments.

Development of PCR-RFLP tests

PCR-RFLP tests were developed to confirm polymorphism in the different NCTC 11168 isolates and to screen a collection of *C. jejuni* GBS/MFS-associated and control strains. PCR-RFLP tests could only be developed for the markers of which the localization on the *C. jejuni* genome was identified. Forward and reverse primers were designed (Primer Designer 4, Sci Ed Software, North Carolina) and synthesized, located approximately 50-200 bp upstream or downstream of the homologous region, respectively (Table 2). Because of the wide range of melting tempera-tures (Tm) of the primers and the sometimes considerable differences in Tm between primers

within one PCR reaction, a touch-down PCR approach was applied. The program consisted of 15 cycles of 1 min 94°C, 1 min 70°C minus 1°C for each following cycle (lowest temperature 55°C), 1 min 72°C, followed by 25 cycles of 0.5 min 94°C, 1 min Tm - 5°C and 1 min at 72°C. Tm – 5°C represents the lowest melting temperature of the two primers used in the reaction minus 5°C. This resulted in the amplification of not only the AFLP fragment itself, but also of their flanking sequences. Next, 15 µl of each PCR product was subjected to a separate or combined digestion with the restriction enzymes (1 unit/reaction) used for the AFLP (*MboI* and *DdeI*). After overnight incubation at 37°C, the digests were analyzed on 2% agarose gels. The PCR-RFLP analysis will reveal whether or not the AFLP variability was due to variation in the restriction sites (different RFLP patterns) or to insertions or deletions within the AFLP fragment (size differences in PCR products). AFLP variation due to differences in the selective extension nucleotides of the AFLP primers will not be detected using this approach.

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Additional Table 1.*C. jejuni* strains used in this study.

strain	associated disease	origin	used for
NCTC 11168-1	enteritis	ID-DLO, Lelystad, The Netherlands (obtained from **NCTC**, UK)	htAFLP, PCR-RFLP
NCTC 11168-2	enteritis	ID-DLO, Lelystad, The Netherlands (obtained from M.B. **Skirrow**, PHLS, UK)	htAFLP, PCR-RFLP
NCTC 11168-3	enteritis	**NRC**, Ottawa, Canada	htAFLP, PCR-RFLP
NCTC 11168-4	enteritis	**UMC** Utrecht, The Netherlands	htAFLP, PCR-RFLP
NCTC 11168-5	enteritis	**LMG** culture collection, Gent, Belgium	htAFLP, PCR-RFLP
NCTC 11168-6	enteritis	**CCUG** culture collection, Göteborg, Sweden	htAFLP, PCR-RFLP
GB1	GBS	The Netherlands	PCR-RFLP
GB2	GBS	The Netherlands	PCR-RFLP
GB3	GBS	The Netherlands	PCR-RFLP
GB4	GBS	The Netherlands	PCR-RFLP
GB5	GBS	The Netherlands	PCR-RFLP
MF6	MFS	The Netherlands	PCR-RFLP
MF7	MFS	The Netherlands	PCR-RFLP
MF8	MFS	The Netherlands	PCR-RFLP
GB11	GBS	The Netherlands	htAFLP, PCR-RFLP
GB13	GBS	The Netherlands	PCR-RFLP
GB14	GBS	The Netherlands	PCR-RFLP
GB15	GBS	The Netherlands	PCR-RFLP
GB16	GBS	Belgium	PCR-RFLP
GB17	GBS	The Netherlands	PCR-RFLP
GB18	GBS	The Netherlands	PCR-RFLP
GB19	GBS	The Netherlands	PCR-RFLP
MF20	MFS	The Netherlands	PCR-RFLP
GB21	GBS	The Netherlands	PCR-RFLP
GB23	GBS	The Netherlands	PCR-RFLP
GB24	GBS	The Netherlands	PCR-RFLP
GB25	GBS	The Netherlands	PCR-RFLP
GB26	GBS	The Netherlands	PCR-RFLP
GB27	GBS	The Netherlands	PCR-RFLP
GB29	GBS	The Netherlands	PCR-RFLP
GB30	GBS	The Netherlands	PCR-RFLP
E97-0737	enteritis	The Netherlands	PCR-RFLP
E97-0747	enteritis	The Netherlands	PCR-RFLP
E97-0796	enteritis	The Netherlands	PCR-RFLP
E97-0873	enteritis	The Netherlands	PCR-RFLP
E97-0903	enteritis	The Netherlands	PCR-RFLP
E97-0921	enteritis	The Netherlands	PCR-RFLP
E97-0974	enteritis	The Netherlands	PCR-RFLP
E97-0980	enteritis	The Netherlands	PCR-RFLP
E97-0998	enteritis	The Netherlands	PCR-RFLP
E97-1013	enteritis	The Netherlands	PCR-RFLP
E98-623	enteritis	The Netherlands	PCR-RFLP
E98-624	enteritis	The Netherlands	PCR-RFLP
E98-682	enteritis	The Netherlands	PCR-RFLP
E98-706	enteritis	The Netherlands	PCR-RFLP
E98-1033	enteritis	The Netherlands	PCR-RFLP
E98-1087	enteritis	The Netherlands	PCR-RFLP
cura 7	GBS	Curaçao, Netherlands Antilles	htAFLP, PCR-RFLP
cura 69	GBS	Bonaire, Netherlands Antilles	PCR-RFLP
cura 276	GBS	Curaçao, Netherlands Antilles	htAFLP, PCR-RFLP
260.94	GBS	South Africa (HS:41)	htAFLP, PCR-RFLP
233.94	GBS	South Africa (HS:41)	htAFLP
233.95	GBS	South Africa (HS:41)	htAFLP
308.95	GBS	South Africa (HS:41)	htAFLP
367.95	GBS	South Africa (HS:41)	htAFLP
370.95	GBS	South Africa (HS:41, same patient as 367.95)	htAFLP
242.98	MFS	South Africa (HS:41)	htAFLP
378.96	enteritis	South Africa (HS:41)	htAFLP
386.96	enteritis	South Africa (HS:41, same patient as 378.96)	htAFLP
199.97	enteritis	South Africa (HS:41)	htAFLP
250.97	enteritis	South Africa (HS:41)	htAFLP
242.97	enteritis	South Africa (HS:41)	htAFLP
21.97	enteritis	South Africa (HS:41)	htAFLP

Additional Table 2. Primer sequences used in the PCR-RFLP analysis for the validation of potential GBS markers.

Marker nr	primers
2	5′-CCTGATCATCTTTCTTGGCATGG-3′
	5′-AAGATCTACACCCTTATCATCTCC-3′
3	5′-AGAAGTGTATTAACAACCTTGC-3′
	5′-ATCATACCGATAATCATCAAAGG-3′
5,6	5′-AGCCACTCAAGCAAATACTAC-3′
	5′-AATAAGGAGCACCATTTAAGG-3′
7	5′-AAGATTATTGGCGATAATCC-3′
	5′-ATAGATACTATCAGCACTCGC-3′
8	5′-GTTATTTCAAGCATCATAGTCG-3′
	5′-ATTTGTCAAAGAATTAGCTCG-3′
9	5′-GCATTAGAAAGTTGCATTAACC-3′
	5′-TTCTTCGCAAGCATTAAGTTC-3′
11	5′-GATGGAGCCAAAGAGCTTGTG-3′
	5′-CACTTGCAGCAGATAAAGCCG-3′
12	5′-GTCAAAGGCGTTCGGATG-3′
	5′-AGCATTGATATGATCAATAGC-3′
13	5′-GCTATTGATCATATCAATGCT-3′
	5′-ATCTTCTTTACTATGATAACTCAC-3′
14	5′-GGGTGATATTTCATATCTTGG-3′
	5′-GCATAAGCTAAATCCTGTCC-3′

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CHAPTER 9 General discussion

- LOS gene polymorphism and LOS structure in the pathogenesis of GBS
- Non-LOS-related factors in the pathogenesis of GBS
- Directions for future research
- Conclusions

GBS is often preceded by an infection with *C. jejuni*. Because only 0.2 – 1 per 1000 symptomatic *C. jejuni* infections are followed by GBS, it is likely that both pathogen-related and host-related factors are important for the development of neurological symptoms. The studies described in this thesis were aimed at the identification of pathogen-related genetic factors.

Molecular mimicry between *Campylobacter* LOS and gangliosides in human nerves is the probable causative mechanism for the development of GBS. Therefore, we first focused on the role of *Campylobacter* LOS biosynthesis genes in the development of GBS. Variation in LOS genes was studied in relation to LOS structures, anti-ganglioside antibody responses and clinical features. In addition, the presence of potential *Campylobacter*-related GBS markers located outside the LOS biosynthesis genes was investigated.

In this section the results described in this thesis will be discussed. The potential role of non-*Campylobacter*-related factors in GBS will also be addressed briefly. Finally, some directions for future research will be given.

LOS gene polymorphism and LOS structure in the pathogenesis of GBS and MFS

Variation in gene content

At least five genetic mechanisms contribute to the expression of different LOS outer core structures, including ganglioside mimics, by *C. jejuni* (Table 2, Introduction). Variation in the content of LOS biosynthesis genes is one of these mechanisms. The LOS biosynthesis genes are clustered in the LOS locus. Variation in gene content is clearly reflected by the different classes of the LOS locus. So far, nine different classes have been identified, including the 4 new LOS classes described in this thesis (1)(Chapter 2 and 6). Characterisation of the LOS biosynthesis genes in additional *C. jejuni* strains will probably result in the identification of additional LOS locus classes. To investigate whether particular genes or combinations of genes involved in LOS biosynthesis are associated with ganglioside mimics or neuropathy, we studied both individual LOS biosynthesis genes (Chapter 3) and the class of LOS locus (Chapter 2) in *C. jejuni* strains isolated from patients with GBS and MFS and from patients without neurological symptoms.

In Chapter 2, we showed that the class A and B LOS loci are associated with the expression of ganglioside mimics and with neuropathy. Nachamkin et al. found a strong association between GBS-associated *C. jejuni* isolates and the simultaneous presence of three LOS biosynthesis genes, *cst-II*, *cgtA* and *cgtB* (2). This is consistent with our findings, as these three genes are only present in class A and B LOS loci. Comparison of the LOS loci of various *C. jejuni* strains has demonstrated that only the class A, B and C LOS loci contain the genes that are necessary for the biosynthesis of ganglioside mimics (1, 3)(Chapter 3). Our observation that the class A and B LOS loci are associated with neuropathy is, therefore, consistent with the ganglioside mimicry hypothesis. However, it is important to realise that more than half of the control *C. jejuni* strains (isolated from patients with enteritis but without neurological symptoms) still have a neuropathy-related LOS locus. This is suggestive for an important role of host-related factors

or other pathogen-related factors in the development of post-infectious neuropathy (Chapter 2)(4).

We found that the class A LOS locus is associated with GBS and GM1-like mimicry and the class B locus with MFS and GQ1b-like mimicry (Chapter 2). GBS has previously been associated with antibodies against monosialylated gangliosides (GM1, GD1a) and MFS with antibodies against disialylated gangliosides (GQ1b) (5-7). Therefore, we hypothesize that there may be a difference between the class A and B LOS locus that determines whether the LOS is mono- or disialylated. There is only one difference in gene content between the class A and B LOS loci: a duplication of *cgtA*, encoding a β-1,4-GalNAc transferase, in the class B locus. Because *cgtA* is not directly involved in the sialylation of the LOS outer core, it is unlikely that this difference is responsible for the observation that class A is associated with GBS and class B with MFS. Recently, Koga et al. confirmed the association of the class A LOS locus with GBS but not of the class B LOS locus with MFS, in a collection of *C. jejuni* strains from Japanese patients (4). Whereas all our MFS strains had a class B LOS locus, the class A and B LOS loci were equally represented in the Japanese MFS strains. This discrepancy may be based on geographical differences between *C. jejuni* strains, but it also has to be taken into account that the Dutch collection comprised only 4 MFS-associated strains.

Although most class C strains express monosialylated ganglioside mimics in their LOS, the class C LOS locus was not more prevalent in GBS-associated strains (Chapter 2)(3, 4). Studies described in this thesis and by Koga et al. showed that most class A strains but no class C strains express a mixture of GM1a and GD1a mimics (Chapter 6) (4). It is currently not known why class C strains do not express GD1a mimics in addition to GM1a mimics. Further research is also needed to determine whether the GM1a/GD1a mixture is more effective than single ganglioside mimics for developing GBS.

We also studied the presence of the individual genes of the class A, B and C LOS loci in a collection of neuropathy-associated and control *C. jejuni* strains by PCR and hybridization analysis (Chapter 3). The gene content as determined by PCR and hybridization was largely in accordance with the expected gene content based on the type of LOS locus. We found that *orf11* was associated with neuropathy. Because *orf11* is unique to the class A and B LOS loci, this observation confirmed our findings that these LOS loci are associated with neuropathy (Chapter 2).

In Chapter 4 we provided evidence by DNA sequence analysis and genotyping techniques that a *C. jejuni* strain can acquire a GBS-associated class A LOS locus through horizontal exchange of the entire LOS locus. Recently, this phenomenon was also demonstrated at a high rate *in vitro* (8). However, it is not known at what frequency this happens *in vivo*, because several requirements have to be met, such as a transformable acceptor strain and coinfection or colonization of the host with different *C. jejuni* strains. Several studies suggest that in humans co-infections with different *C. jejuni* strains occur in a frequency of approximately 7-8%, whereas most chickens are colonized with multiple *Campylobacter* strains (Chapter 5)(9, 10).

LOS genes and the induction of an anti-ganglioside antibody response

Based on the association of the class A and B LOS loci with neuropathy and the expression

of ganglioside mimics, we hypothesized that genes unique to the class A and B LOS loci or genes involved in sialylation of the LOS are potential GBS markers. *Orf11* is the only gene that is unique to the class A and B loci (Figure 3, Introduction). It encodes a putative acetyltransferase. Three LOS biosynthesis genes are known to be involved in sialic acid biosynthesis: *orf8* (*neuB*), *orf9* (*neuC*) and *orf10* (*neuA*) (Figure 2, Introduction). *Orf7* (*cst-II*) is involved in the transfer of sialic acid to the LOS backbone. To investigate whether these potential GBS marker genes are important for the expression of ganglioside mimics and induction of an anti-ganglioside antibody response, we inactivated *orf7*, *orf10* and *orf11* by the construction of three different knock-out mutants. We did not make knock-out mutants of *orf8* and *orf9*. Because these genes are also involved in sialic acid biosynthesis, it is expected that the inactivation of *orf8* and *orf9* would have the same effect as inactivation of *orf10* .

There were no detectable differences in LOS structure or antibody response in mice when the *orf11* knock-out mutant was compared with the wild-type. Thus, we did not demonstrate a role of this gene in the biosynthesis of the LOS and pathogenesis of GBS. Very recently, Houliston et al. demonstrated that the product of *orf11* catalyzes the transfer of an O-acetyl-group to oligosaccharide-bound sialic acid, with a strong preference for α-2,8-linked sialic acid (11). This observation does not indicate a role of *orf11* in GBS, because only disialylated LOS contains α-2,8-linked sialic acid. In addition, the product of *orf11* was predicted to be inactive in several neuropathy-associated strains with disialylated LOS, rendering a role of this gene in GBS or MFS even more uncertain.

Inactivation of either *cst-II* or *orf10* resulted in the expression of truncated LOS without sialic acid. The truncated LOS showed reduced reactivity with GBS patient serum and failed to induce an anti-ganglioside antibody response in mice, in contrast to the wild-type LOS. Therefore, we concluded that genes involved in sialylation of the LOS are crucial for the induction of anti-ganglioside antibodies. Recently, similar results were obtained with a knock-out mutant of *neuB*, encoding a sialic acid synthetase (12) and with a knock-out mutant of *galE*, a gene located in the *pgl* gene locus (13). Inactivation of *galE* probably results in a heavily truncated LOS core without galactose residues and, consequently, also no sialic acid residues (14).

Polymorphism within LOS genes

Strains with the same LOS locus express different LOS structures due to DNA polymorphism within genes, such as polynucleotide tracts with phase variation, single nucleotide deletions and single or multiple nucleotide mutations (15). In Chapter 3, we studied sequence heterogeneity within class A, B and C LOS biosynthesis genes by PCR-RFLP analysis. We did not find an association between a particular RFLP type and specific ganglioside mimics or neuropathy. We concluded that it is probably necessary to perform more detailed DNA sequence analysis to identify polymorphisms within genes that are important for ganglioside mimicry and neuropathy.

In Chapter 5 and 6 we used DNA sequence analysis to study the relation between polymorphism within genes and LOS structure in GBS/MFS-associated strains. We showed that, as a result of polymorphism within genes, 3 out of 23 strains (13%) with a class A, B or C LOS

locus fail to express ganglioside mimics in their LOS. In addition, our results suggested that the expression of ganglioside mimics by a *C. jejuni* strain may disappear during an infection or during subculture, which has important implications for the analysis of strains isolated from GBS patients and interpretation of results.

Polymorphism within the *cst-II* gene determines whether the LOS is mono- or disialylated (15). Most of the strains with a Thr51 allele express monosialylated LOS, whereas most strains with an Asn51 allele express disialylated LOS. We showed that the Asn51-allele was present in most strains associated with MFS and GBS with ophthalmoplegia, whereas Thr51 was the most prevalent allele in the other GBS-associated strains (Chapter 6). Koga et al. demonstrated that the Thr51 allele is associated with GBS, whereas the Asn51 allele is associated with MFS (16).

Ganglioside mimics in neuropathy-associated C. jejuni strains

Initially, we used serological methods to identify ganglioside-like structures in the *C. jejuni* LOS. More recently, the availability of new mass spectrometry methods enabled us to determine the exact LOS structures in all our neuropathy-associated strains (Chapter 6).

We detected a large variety in LOS outer core structures and showed that most strains express a mixture of different outer core structures. Interestingly, 36% of GBS-associated strains expressed a mixture of GM1a- and GD1a-like LOS. All strains with the GM1/GD1a-like LOS carried the class A LOS locus, which was previously found to be associated with GBS (Chapter 2) (4). This finding suggests that a cluster or complex of the GM1a/GD1a ganglioside mimics, which is only expressed by strains with a class A LOS locus, may be the primary target antigens in a subgroup of GBS, rather than single ganglioside mimics.

Most strains isolated from patients with ophthalmoplegia (MFS or GBS) expressed disialylated (GD1c-like) LOS. This observation suggests that in these patients, pathogenic antibodies are raised against the GD1c-like LOS and cross-react with ganglioside GQ1b, which is enriched in the nerves that innervate the oculomotor muscles. True GQ1b-like structures (which contain 4 sialic acid residues) were not detected in the LOS.

Recent studies showed that some GBS and MFS patients produce antibodies to complexes of gangliosides rather than to single gangliosides (17) (18). In these cases, the antibody reactivity against the complex of two gangliosides is higher than the reactivity against the single gangliosides. Our observation that many GBS- and MFS-associated strains express of a mixture of ganglioside mimics in their LOS, led to the hypothesis that *C.jejuni* LOS may induce antibodies against ganglioside complexes. In Chapter 7 we investigated the presence of ganglioside complex antibodies in patient sera and related these antibodies to the LOS structures of the corresponding *C. jejuni* strains. Antibodies against ganglioside complexes GM1a/GD1a and GQ1b/GD1a were demonstrated in serum samples from 4 out of 26 *C. jejuni*-related GBS patients. The *C. jejuni* strains isolated from the patients with anti-GM1a/GD1a antibodies expressed LOS with a GM1/GD1a mixture and LOS with an asialo-GM1/GM1b/GD1c mixture. However, the *C. jejuni* strains isolated from the patients with anti-GQ1b/GD1a antibodies expressed a homogeneous LOS with only a GD1c mimic. Despite of the observed discrepancies between LOS structures and antibody specificities, the ganglioside complex antibodies

cross-reacted with the LOS of the corresponding *C. jejuni* strains in all four patients. These findings suggest that the ganglioside complex antibodies were primarily induced by the *C. jejuni* LOS and that conformational epitopes may be shared by different oligosaccharides in LOS and ganglioside complexes.

Mass spectrometry showed that 6/22 (27%) of GBS-associated strains did not express ganglioside mimics. However, absence of ganglioside mimics in a strain isolated from a GBS patient does not necessarily imply that ganglioside mimicry was not the causative mechanism for GBS in that patient. In Chapter 5 we showed that co-infection with different *C. jejuni* strains occur in GBS patients. In such cases, it is possible that a ganglioside-mimicking *C. jejuni* strain induces GBS in a patient, but that only a co-infecting strain without ganglioside mimics is isolated from the stool sample and wrongfully regarded as the strain that caused GBS. In addition, it is possible that the expression of ganglioside mimics disappears later in the course of infection or during subculture after isolation of the *C. jejuni* strain from the faeces. Indeed, we found that two GBS-associated strains did not express ganglioside mimics due to a mutation or deletion in one of the LOS genes, which may have been introduced during the infection of the GBS patient of during subculture (Chapter 6). In conclusion, we confirmed that ganglioside mimicry is the most likely pathogenic mechanism underlying the majority of *C. jejuni*-associated GBS cases.

Neuropathy-associated C. jejuni strains without ganglioside mimics

The results presented in this thesis indicate that occasionally, mechanisms other than mimicry of *C. jejuni* LOS with gangliosides may also cause GBS. Possible mechanisms include mimicry with *C. jejuni* LOS without ganglioside mimics or with *C. jejuni* structures other than LOS, mimicry with other micro-organisms or mechanisms not involving mimicry. In Chapter 5 we showed that serum of a GBS patient contained anti-asialo-GM2 antibodies cross-reactive with the asialo-GM2-like LOS of the corresponding *C. jejuni* strain. This finding suggests that molecular mimicry with non-sialylated LOS may have led to development of GBS. There were two other cases in which the *C. jejuni* strains lacked ganglioside mimics and the corresponding patient sera did not contain anti-ganglioside antibodies. This finding again suggests a pathogenic mechanism other than ganglioside mimicry (Chapter 6) and further investigations are needed to elucidate the pathogenesis of GBS and the role of *C. jejuni* or other microbial species in these cases.

Non-LOS related factors in the pathogenesis of GBS

Non-LOS-related Campylobacter factors

Associations between the expression of ganglioside mimics and neuropathy described in this thesis and by other groups are not absolute. Apart from host factors (see below), other *Campylobacter*-related factors may also contribute to the neuropathogenic potential of a strain. However, up to now, the role of such factors in the development of GBS has not been established.

HS:19 and HS:41 serotypes have been associated with GBS (19, 20). Because the capsule is the determinant of the HS serotype (21), these observations would suggest a potential role for

the capsule in GBS. However, our findings indicate that the association between serotype and GBS can also be based on the presence of a class A LOS locus in these highly clonal populations (Chapter 2). Limited data about presence and heterogeneity of genes involved in capsule biosynthesis do not show an association of these genes with GBS (22) (P. Godschalk, unpublished observations). Additional studies are needed to establish the mechanism underlying the association between serotype and GBS.

We used a high-throughput AFLP approach to search for potential new GBS factors in *C. jejuni* (Chapter 8). Ht-AFLP screens the genome in a random fashion with a very high resolution when compared to other molecular typing techniques. The results of the ht-AFLP confirmed that LOS genes are involved in GBS, but we did not find GBS markers outside the LOS gene locus. Other studies aiming at the detection of potential non-LOS GBS factors have been unsuccessful as well, rendering the existence of such factors uncertain (22-26).

Host-related and environmental factors

The importance of host and environmental factors in the susceptibility for developing GBS after a *C. jejuni* infection is favored by several observations. The most important observations, but certainly not all, will be discussed next.

More than half of all *C. jejuni* strains isolated from human feces express ganglioside mimics, whereas it is estimated that GBS occurs after only 1 in 1000-3000 *C. jejuni* infections (27, 28). In addition, family outbreaks of diarrea due to infection with the same *C. jejuni* strain resulted in only one case of GBS in each family (29, 30). On the other hand, occurrence of GBS within families has been described (though not after a simultaneous infection with one *C. jejuni* strain), which is suggestive for genetic factors in development of the disease (31). It has also been demonstrated that the risk for recurrence of GBS is higher than the risk to develop GBS for the first time (32).

Thus, the "right" combination of pathogen- and host-related factors is probably necessary for the development of GBS. In addition, interactions between these factors and/or environmental factors may also be important. For example, it is possible that the expression of ganglioside mimics by *C. jejuni* in the gut is influenced by local conditions and by the interaction of *C. jejuni* with host epithelial cells, other bacteria and/or the host's immune system. A study with human volunteers indicated that *Campylobacter* isolates had undergone antigenic phase variation in ganglioside mimicry during passage *in vivo* (33). The host´s immune response may also be influenced by environmental factors such as exposition to other micro-organisms (e.g. gut flora). Additional research is needed to further elucidate the complex interaction between host, pathogen and environment in the development of GBS.

Although there are some conflicting results, most studies performed so far do not support a role of host immunogenetic factors in conferring susceptibility to develop GBS (34-36). However, identification of such factors may be extremely difficult due to the complex contribution of a combination of pathogen-related factors, different host polymorphisms and interactions with the environment. Several polymorphisms in genes involved in immune response have been associated with the presence of cross-reactive antibodies in serum of GBS patients and with severity of disease and outcome (36).

Directions for future research

LOS structure and GBS

We used mass spectrometry analysis to determine the exact (mixture of) LOS structures in a large collection of neuropathy-associated *C. jejuni* strains. These data provide the opportunity to relate LOS structure to detailed clinical data (e.g. specificity of anti-ganglioside antibody responses, symptoms, prognosis) more accurately than in previous studies, when presence of ganglioside mimics in *C. jejuni* LOS was determined with serological methods only. Mass spectrometry analysis of additional neuropathy-associated strains from different geographical regions and of control strains may further expand our insight into the relationship between LOS structure and clinical symptoms.

Regulation of LOS expression

In this thesis the relationship between LOS biosynthesis genes, LOS structures and development of GBS or MFS was studied. The relative and absolute amounts of the expressed (mixtures of) LOS structures may vary between strains and between different growth and environmental conditions. It is possible that the expression of high amounts of a ganglioside mimic is crucial for triggering GBS. Therefore, it would be interesting to study the regulatory mechanisms of LOS expression. Differences in the levels of mRNA expression between different strains or growth conditions can be determined with micro-array (gene chips) or RT-PCR techniques. Unfortunately, to our knowledge, there are no straightforward and easy techniques to quantify the relative and absolute amounts of LOS structures that are expressed by a strain under different growth circumstances.

Host-pathogen interaction

Studying host-pathogen interactions on a molecular level may provide a better understanding of the pathogenesis of GBS. The host environment and interaction with host cells may influence or modulate the expression of ganglioside mimics by *C. jejuni*. As mentioned in the previous paragraph, gene chips can be used to study the expression of genes involved in ganglioside mimicry in relation to the host, e.g. by using human cell lines or an *in vivo* situation, such as an animal model. The potential role of other *Campylobacter*-related factors in GBS may also be identified using this approach.

On the other hand, the host's response to different *Campylobacter* strains, both neuropathy-associated and control strains, should also be studied. The *Campylobacter* knock-out strains described in this thesis provide an excellent opportunity to study the influence of LOS sialylation on host responses. Microarrays containing host genes can be used to detect differences in gene expression after exposure of a human cell line or animal to *C. jejuni*. Measurement of various cytokines in reaction to adherence of or invasion with different *Campylobacter* strains may provide a better insight into the initiation of the immune response that ultimately leads to neurological damage.

Other Campylobacter factors

Several studies have unsuccessfully tried to identify *Campylobacter*-related GBS factors besides the LOS. The availability of new and more sensitive geno- and phenotyping techniques may warrant some additional studies. However, the existence of such non-LOS GBS factors is uncertain and this should be considered before undertaking even more extensive studies than already performed. Although whole genome sequencing would be the most sensitive genotyping technique for the detection of GBS factors, this technique is not (yet) feasible for the comparison of larger collections of strains. New micro-arrays which contain an extended number of genes have become available and may result in the detection of additional GBS markers. However, GBS-factors that are not based on the differential presence or absence of a gene will not be detected with such a microarray analysis. Micro-arrays can also be used to detect differences in mRNA expression levels. GBS factors that result in differential expression of mRNA may be detected with this approach. Comparison of the proteomes (protein expression) or glycomes (polysaccharide expression) may also be used to search for additional GBS factors. However, preliminary studies failed to detect an association between *Campylobacter* proteins and GBS (P. Godschalk, unpublished observations).

Some GBS patients did not have an anti-ganglioside antibody response and were infected with a *C. jejuni* strain without ganglioside mimics (Chapter 6). Mechanisms other than ganglioside mimicry may have played a role in the pathogenesis of GBS in these patients. Further analysis of such *C. jejuni* strains may result in the detection of new GBS factors that played a role in these patients and that may be important in other GBS patients as well.

LOS structure and pathogenicity in general

Ganglioside mimicry is involved in the pathogenesis of post-infectious neuropathy. Sialylation of LOS may also be involved in other aspects of *Campylobacter* infection. Determination of the LOS locus class is straightforward and a good (although not 100% accurate) predictor for the presence of ganglioside mimics. Epidemiological studies to determine associations between certain LOS classes and specific geographical regions, certain hosts (human, animal) or clinical features (severity of gastroenteritis, reactive arthritis, immune response) can be performed and may provide indications for the role of LOS in the pathogenesis of *Campylobacter* infections in general.

The role of LOS structure in the pathogenesis of *Campylobacter* infections (e.g. virulence) should be further studied by functional *in vitro* and/or *in vivo* studies, also using the knock-out mutants described in this thesis.

Treatment of GBS

Despite optimal medical care, GBS-associated mortality remains at approximately 5% and a considerable proportion of patients suffer from severe residual deficits. Thus, better treatment options are necessary. A promising new therapeutic approach that is currently being investigated is immunoadsorption therapy (37). In this approach, anti-glycolipid antibodies are eliminated from the circulation through specific immunoadsorption to immobilized oligosaccha-

rides. Considering the large variety in specificity of anti-glycolipid antibodies that is observed in GBS patients, it is crucial to use oligosaccharides with the right structure or combinations of structures for immunoadsorption. It may be possible to develop (a mixture of) oligosaccharides that can effectively bind all anti-glycolipid antibodies in all patients. Otherwise, it may be necessary to determine the optimal adsorbent for every individual patient. In *Campylobacter*-related GBS patients, oligosaccharides that correspond with the structure(s) of the appropriate *Campylobacter* LOS may form the optimal adsorbent, because the anti-ganglioside antibodies are initially raised against the *Campylobacter* LOS and cross-react with gangliosides. Further research addressing this issue is needed. At present, it takes at least 6 days to determine LOS structures, including the isolation of the *C. jejuni* strain from the faeces. It is desirable to start optimal treatment as soon as possible after onset of symptoms. New developments in mass spectrometry techniques may lead to more rapid and straightforward determination of *Campylobacter* LOS structures. More extensive knowledge on the relationship between LOS structure and DNA sequence of LOS biosynthesis genes may eventually eliminate the need for mass spectrometry to predict LOS structure.

Conclusions

- *Current genetic markers for GBS and MFS in C. jejuni are located in the LOS biosynthesis genes.*
  This finding is consistent with the hypothesis of ganglioside mimicry as causative mechanism for GBS after infection with *C. jejuni.* Genetic markers for GBS located outside the LOS biosynthesis gene locus have not been identified so far and it is not certain whether such markers exist.
- *Specific LOS biosynthesis loci are associated with the expression of ganglioside mimics and neuropathy.*
  LOS class A is associated with GBS and monosialylated ganglioside (GM1-like) mimics. LOS class B is associated with MFS and disialylated ganglioside (GQ1b-like) mimics.
- *A GBS-associated LOS locus may be acquired through horizontal gene transfer.*
- *Genes involved in sialylation of the LOS are crucial for the induction of an anti-ganglioside antibody response.*
  Inactivation of a gene involved in sialic acid biosynthesis or transfer resulted in a truncated LOS without sialic acid. Immunization with the mutant bacteria did not induce an anti-ganglioside antibody response in mice, in contrast to immunization with the wild type bacteria.
- *Sialylation of the LOS determines clinical symptoms.*
  Most *C. jejuni* strains isolated from GBS patients without oculomotor symptoms express monosialylated ganglioside mimics. Strains isolated from patients with MFS or GBS with oculomotor symptoms express disialylated (GD3-like) mimics (Figure 1).
- *The cst-II gene sequence determines whether LOS is mono- or disialylated and thereby the clinical symptoms.*

Most of the strains with a Thr51 allele express monosialylated LOS, whereas most strains with an Asn51 allele express disialylated LOS. The association of a class A LOS locus with GBS and class B LOS locus with MFS is probably based on differences in the cst-II sequence that determine whether the LOS is mono- or disialylated (Figure 1).

- *A mixture of GM1a/GD1a ganglioside mimics may be the target antigens in a subgroup of GBS.*
  This mixture was present in 36% of all GBS-associated *C. jejuni* strains, all of which had a class A LOS locus.
- *Antibodies against ganglioside complexes may be induced by C. jejuni LOS.*
  Antibodies against ganglioside complexes cross-reacted with the corresponding *C. jejuni* LOS. However, the LOS structures were similar but not always identical to those in the corresponding ganglioside complexes, suggesting that conformational epitopes may trigger the antibody response in these cases.
- *A C. jejuni strain that is isolated from the faeces of a GBS patient is not necessarily involved in the pathogenesis of GBS in that patient.*
- *In some cases of GBS that is preceded by infection with C. jejuni, a mechanism other than mimicry of C. jejuni LOS with gangliosides may play a role in the development of GBS.*
  Mimicry of *C. jejuni* LOS with the glycolipid asialo-GM2 may induce a cross-reactive anti-glycolipid antibody response leading to GBS.

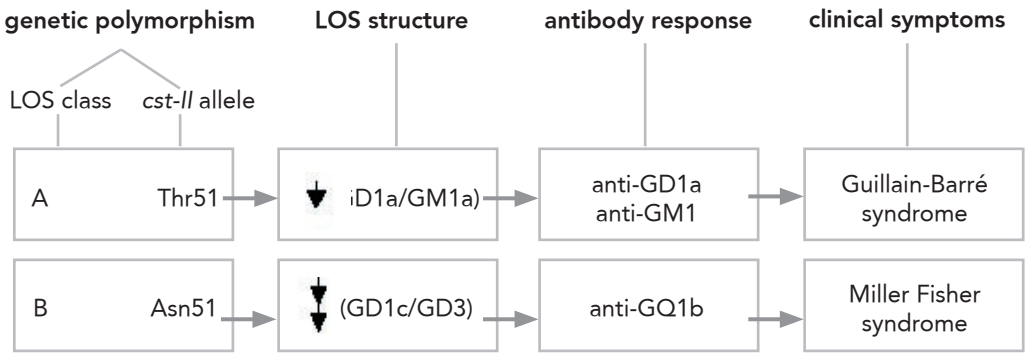

Figure 1. Proposed scheme for the role of genetic polymorphism of *C. jejuni* LOS genes in clinical symptoms of post-infectious neuropathy. Strains with a class A LOS locus often have the cst-II Thr51 allele. These strains can express monosialylated ganglioside mimics (mostly GM1a- and/or GD1a-like) and induce an anti-GM1, anti-GD1a or anti-GM1/GD1a complex antibody response in certain patients. Such an antibody response induces symptoms of GBS. Strains with a class B LOS locus often have the cst-II Asn51 allele. These strains can express disialylated ganglioside mimics (GD1c- and GD3-like) and induce an anti-GQ1b antibody response in certain patients. Such an antibody response induces symptoms of MFS.

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Summary

Samenvatting

Summary

The Guillain-Barré syndrome (GBS) and its variant the Miller Fisher syndrome (MFS) are acute immune-mediated polyneuropathies. GBS and MFS are often preceded by an infection with *Campylobacter jejuni*. There is strong evidence that molecular mimicry between *Campylobacter* lipooligosaccharides (LOS) and gangliosides in nervous tissue induces a cross-reactive anti-ganglioside antibody response leading to nerve damage and clinical symptoms of GBS or MFS.

The aim of this thesis was to identify genetic markers for GBS/MFS in *C. jejuni*. We initially focused on *Campylobacter* genes involved in LOS biosynthesis. We hypothesized that polymorphism of *C. jejuni* LOS biosynthesis genes determines LOS structure and thereby the capability of a *C. jejuni* strain to induce a certain anti-ganglioside antibody response and clinical symptoms of GBS/MFS. Because other pathogen-related factors may be important for the development of GBS/MFS as well, we also aimed at the identification of potential genetic markers for GBS outside the LOS biosynthesis genes.

First, we investigated whether the presence of certain *C. jejuni* LOS biosynthesis genes, or combinations of these genes, are crucial for the induction of anti-ganglioside immune responses that lead to GBS (**Chapter 2**). We developed LOS class-specific PCR tests and determined the type of LOS biosynthesis gene locus in our GBS/MFS-associated and control strains. Although 5 different LOS locus classes had been described so far, only the classes A, B and C contain the right genes to synthesize ganglioside mimics. We found that the class A LOS locus was associated with GBS and GM1-like mimicry, whereas the class B LOS locus was associated with MFS and GQ1b-like mimicry. Based on these findings, we considered genes unique for class A and B LOS loci or genes involved in sialylation of the LOS as potential GBS marker genes. Knockout mutants of two of these genes, *cst-II* and *orf10*, both involved in LOS sialylation, expressed truncated LOS structures without sialic acid, showed reduced reactivity with GBS patient serum and failed to induce an anti-ganglioside antibody response in mice. We concluded that specific genes, involved in sialylation of the LOS, are crucial for the induction of anti-ganglioside antibodies.

In search of other and/or more specific markers for GBS, we studied the presence and heterogeneity of all individual genes within the class A, B and C LOS loci by a comparative PCR-RFLP and hybridization analysis (**Chapter 3**). We confirmed our previous observation that the presence of certain LOS biosynthesis genes is associated with neuropathy and the biosynthesis of ganglioside mimics. RFLP analysis revealed extensive sequence heterogeneity within most LOS genes, but we did not find an association between certain RFLP types and neuropathy or ganglioside mimicry.

In **Chapter 4**, we provided evidence that a *C. jejuni* strain can acquire the GBS-associated class A LOS locus through horizontal exchange of the entire LOS locus. Comparison of the "genome" strain NCTC 11168 with GB11, a GBS-associated strain, showed that they have a different LOS locus whereas the strains were otherwise closely related as determined by various genotyping and serotyping methods. DNA sequence analysis revealed the positions in the

LOS locus where the exchange had most likely occurred. In **Chapter 5**, we demonstrated that molecular mimicry with structures other than sialylated gangliosides may also trigger GBS. We showed that serum of a GBS patient contained anti-asialo-GM2 antibodies cross-reactive with the asialo-GM2-mimicking LOS of the corresponding *C. jejuni* strain. The expression of this truncated LOS was the result of a mutation in one of the LOS biosynthesis genes. In addition, we demonstrated that co-infections with different *C. jejuni* strains occur in GBS patients. This finding has important implications for the analysis of *C. jejuni* strains isolated from GBS patients, because a strain isolated from a GBS patient is not necessarily the strain that triggered GBS in that patient.

The availability of mass spectrometry techniques combined with DNA sequence data provided us with the unique opportunity to determine the exact LOS outer core structures of our collection of GBS- and MFS-associated strains (**Chapter 6**). Different genetic mechanisms were responsible for the large variety in LOS structures. Many strains expressed a mixture of ganglioside mimics. A mixture of GM1a/GD1a mimics was frequently observed in GBS-associated strains with a class A LOS locus. Most GBS-associated strains expressed mono-sialylated LOS, whereas all MFS-associated strains and 75% of strains from GBS-patients with ophthalmoplegia expressed di-sialylated LOS. Whether strains express mono- or disialylated LOS is determined by the sequence of the *cst-II* gene. Several GBS-associated strains did not express LOS with ganglioside mimics and our data suggest that in some of these cases, mechanisms other than molecular mimicry of *C. jejuni* LOS with gangliosides may have triggered GBS. In addition, we identified two new LOS locus classes. In **Chapter 7**, we demonstrated that antibodies against ganglioside complexes may be primarily induced by *C. jejuni* LOS. In addition, we found that different oligosaccharide moieties in the LOS and the associated ganglioside complexes may form similar conformational epitopes. In **Chapter 8**, we used a new high-throughput AFLP (htAFLP) approach to search for novel potential GBS markers throughout the *Campylobacter* genome. We compared three GBS-associated *C. jejuni* strains with a control *C. jejuni* strain by htAFLP and the resulting potential GBS-markers were further evaluated by a PCR-RFLP analysis of a larger collection of neuropathy and control strains. In addition, we analysed a collection of highly clonal South-African GBS-associated and control strains. We did not find any GBS-specific markers, although three markers, all located in the LOS biosynthesis locus, were significantly associated with GBS. These data confirmed the current view that mimicry with LOS is the prime etiologic determinant of GBS.

In conclusion, the data presented in this thesis show that genetic markers for GBS in *C. jejuni* are located in the LOS biosynthesis genes. Different genetic polymorphisms determine the variation in LOS structure of neuropathy-associated *C. jejuni* strains. Certain polymorphisms, such as type of LOS locus and *cst-II* gene sequence, are associated with specific ganglioside-like LOS structures and clinical features. Our data confirm that ganglioside mimicry is the most likely causative mechanism in most cases of *C. jejuni*-related GBS. However, they also suggest that, in some cases, *C. jejuni*-related GBS may be triggered by mechanisms other than mimicry of *C. jejuni* LOS with gangliosides.

Samenvatting (ook voor niet-ingewijden)

Het Guillain-Barré syndroom (GBS) is een zeldzame aandoening van het perifere zenuwstelsel. Deze aandoening wordt gekenmerkt door een snel optredende zwakte of verlamming van de spieren. GBS wordt vaak voorafgegaan door een infectie met de bacterie *Campylobacter jejuni*. *Campylobacter* is ook de belangrijkste bacteriële verwekker van infectieuze diarree wereldwijd. Eerder onderzoek heeft aannemelijk gemaakt dat GBS wordt veroorzaakt door een structurele gelijkenis ("moleculaire mimicry") tussen enerzijds lipo-oligosacchariden (LOS) in de celwand van de *Campylobacter*-bacterie en anderzijds gangliosiden in de menselijke perifere zenuwvezels. In sommige gevallen maakt het immuunsysteem antistoffen aan die gericht zijn tegen het LOS van de bacterie. Door de gelijkenis in structuur echter kunnen deze antistoffen kruisreageren met de gangliosiden in de zenuwvezels van de patiënt. Zo kunnen ze de zenuwvezels beschadigen en treedt er verlamming op. Er zijn veel verschillende gangliosiden; van bepaalde gangliosiden is het bekend dat er antistoffen tegen gemaakt worden die geassocieerd zijn met specifieke klinische symptomen. Zo worden antistoffen tegen ganglioside GQ1b vaak gevonden bij patiënten met het Miller-Fisher syndroom (MFS). MFS is een variant van GBS die wordt gekenmerkt door verlamming van de oogbolspieren, dronkenmansgang en afwezigheid van reflexen.

In dit proefschrift is onderzocht welke genetische factoren van *C. jejuni* belangrijk zijn voor het optreden van GBS of MFS. Vanwege de "moleculaire mimicry" hypothese hebben we ons in eerste instantie gericht op de genen die betrokken zijn bij de aanmaak van het LOS. We hebben onderzocht welke factoren de structuur van het LOS bepalen. Ook hebben we onderzocht of bepaalde factoren geassocieerd zijn met een specifieke anti-ganglioside antistofrespons en met specifieke symptomen van GBS. Naast het LOS kunnen ook nog andere eigenschappen van de *Campylobacter* belangrijk zijn voor het optreden van GBS of MFS. Daarom hebben we ook naar genetische factoren buiten de LOS-genen gezocht.

Onze eerste vraag was of een van de genen betrokken bij de biosynthese van LOS (of combinaties daarvan) aanwezig moeten zijn om een anti-ganglioside antistofrespons op te wekken die leidt tot GBS (**Hoofdstuk 2**). De genen die betrokken zijn bij de LOS-biosynthese liggen bij elkaar gegroepeerd in het zogenaamde "LOS-biosynthese locus". De verschillende bekende combinaties van LOS-biosynthese genen worden aangeduid als LOS-klasse A, B, C, enzovoort. Bepaling van de LOS-klasse in al onze GBS-geassocieerde en controlestammen liet zien dat klasse A is geassocieerd met GBS en GM1-achtig LOS, terwijl klasse B geassocieerd is met MFS en GQ1b-achtig LOS. Op basis van deze bevindingen veronderstelden we het volgende: genen die uniek zijn voor de klasse A en B LOS-loci, of genen die betrokken zijn bij de sialylering van het LOS, zijn potentiële markers voor GBS.

Hierna hebben we knock-out mutanten gemaakt van diverse van deze genen. Dit zijn *C. jejuni*-bacteriën waarin een bepaald gen kunstmatig is uitgeschakeld. Met behulp van deze knock-out mutanten hebben we laten zien dat het uitschakelen van de genen *cst-II* of *orf10* ertoe leidt dat het LOS sterk verminderd reageert met antistoffen in patiëntenserum. Zowel *cst-*

II als *orf10* zijn betrokken bij de sialylering van het LOS. Ook waren deze knock-out mutanten niet in staat om een anti-ganglioside antistofrespons op te wekken in muizen. Dit lukte de wild-type bacteriën (met actief gen) wél. Hiermee hebben we aangetoond dat specifieke genen die betrokken zijn bij de sialylering van LOS cruciaal zijn voor het opwekken van een potentieel neuropathogene anti-ganglioside antistofrespons.

Op zoek naar andere of meer specifieke GBS-markers binnen de LOS-biosynthese genen, hebben we de aanwezigheid van de individuele genen van de klasse A, B en C LOS-loci onderzocht. Ook hebben we gekeken naar de variatie in DNA-sequentie in die genen. Dit deden we met behulp van PCR-RFLP- en hybridisatietechnieken (**Hoofdstuk 3**). De resultaten bevestigen de in Hoofdstuk 3 gevonden associaties. Ze laten bovendien zien dat er veel variatie in DNA-sequentie ("polymorfisme") is binnen de meeste LOS-genen. Er werd echter geen associatie gevonden tussen een bepaald polymorfisme en GBS/MFS of een ganglioside-achtige LOS-structuur.

In **Hoofdstuk 4** maken we aannemelijk dat een *C. jejuni*-stam een GBS-geassocieerd klasse A LOS-locus kan verkrijgen door DNA uit te wisselen met een andere bacterie ("horizontal exchange"). We vergeleken de *C. jejuni* "genoom"stam NCTC 11168 met GB11, een stam die is geïsoleerd uit de ontlasting van een GBS-patiënt. Hieruit bleek dat deze stammen een verschillend LOS-locus hebben, terwijl ze verder genetisch vrijwel identiek zijn. Met behulp van DNA-sequentieanalyse werden de plaatsen in het LOS-locus vastgesteld waar deze uitwisseling van DNA waarschijnlijk heeft plaatsgevonden.

Onderzoek beschreven in **Hoofdstuk 5** laat zien dat GBS mogelijk ook veroorzaakt kan worden door moleculaire mimicry met structuren die anders zijn dan siaalzuurbevattende gangliosiden. Het serum van een patient met GBS bevatte anti-asialo-GM2 antistoffen die kruis-reageerden met het asialo-GM2-achtige LOS van de *Campylobacter*-stam die uit de ontlasting van de betreffende patiënt was geïsoleerd. De expressie van dit verkorte asialo-GM2-achtige LOS was het resultaat van een mutatie in één van de LOS-biosynthese genen. Daarnaast toonden we aan dat GBS-patiënten tegelijk een co-infectie met verschillende *Campylobacter*-stammen kunnen hebben. Deze bevinding heeft consequenties voor onderzoek naar GBS-geassocieerde *Campylobacter*-stammen: een stam die geïsoleerd is uit de ontlasting van een GBS-patiënt is namelijk niet noodzakelijk dezelfde stam die GBS heeft veroorzaakt bij die patiënt.

De beschikbaarheid van massa-spectrometrietechnieken gecombineerd met DNA-sequentiegegevens maakte het mogelijk om van al onze GBS/MFS-geassocieerde stammen de exacte LOS-structuur te bepalen (**Hoofdstuk 6**). Hieruit bleek dat de grote variatie in LOS-structuren veroorzaakt wordt door verschillende genetische mechanismes. De meerderheid van de GBS-geassocieerde stammen heeft een LOS met een keten van maximaal één siaalzuur, terwijl de meeste stammen van patiënten met oogbolmotoriekstoornissen (MFS en soms GBS) een LOS met een keten van twee siaalzuurgroepen hebben. De DNA-sequentie van het *cst-II*-gen bepaalt het aantal siaalzuurgroepen in de keten. Het was opvallend dat sommige GBS-geassocieerde stammen helemaal geen LOS met ganglioside-achtige structuren tot expressie brachten. Onze gegevens suggereren dat in enkele van deze gevallen de oorzaak van GBS mogelijk een ander

mechanisme is dan mimicry van *Campylobacter*-LOS met gangliosides.

In **Hoofdstuk 7** wordt onderzoek gepresenteerd waaruit blijkt dat *Campylobacter*-LOS waarschijnlijk ook antistoffen kan opwekken die gericht zijn tegen een mengsel van twee verschillende gangliosiden. Veel *Campylobacter*-stammen hebben een LOS met meerdere ganglioside-achtige structuren.

In **Hoofdstuk 8** is een nieuwe high-throughput AFLP (htAFLP) techniek gebruikt om het *Campylobacter*-genoom te screenen op nieuwe GBS-markers. Hiervoor zijn drie GBS-geassocieerde stammen vergeleken met een controlestam. De resulterende potentiële GBS-markers hebben we verder geëvalueerd in een grotere stammencollectie met behulp van een PCR-RFLP-analyse. Daarnaast is er een genetisch zeer verwante collectie van Zuid-Afrikaanse GBS-geassocieerde en controlestammen met htAFLP geanalyseerd. Er zijn geen GBS-specifieke markers gevonden, hoewel drie markers, alle gesitueerd in het LOS-locus, significant geassocieerd waren met GBS.

Het onderzoek beschreven in dit proefschrift laat zien dat genetische markers voor GBS en MFS in de LOS-biosynthese genen liggen. Verschillende genetische polymorfismen bepalen de variatie in LOS-structuur in *C. jejuni*. Bepaalde polymorfismen, zoals de klasse van het LOS-locus en de sequentie van het *cst-II*-gen, zijn geassocieerd met specifieke ganglioside-achtige LOS-structuren en met specifieke klinische symptomen. Onze bevindingen bevestigen dat mimicry van LOS met gangliosiden het meest waarschijnlijke mechanisme is voor het optreden van GBS of MFS. Maar ze suggereren ook dat het ontstaan van GBS na een *Campylobacter*-infectie in sommige gevallen het gevolg is van een ander mechanisme.

Dankwoord

Dankwoord

Het meest gelezen onderdeel van een proefschrift is ook een van de weinige onderdelen dat niet eindeloos is gecorrigeerd door supervisoren, co-auteurs en peer reviewers. Dus ongecensureerd en helemaal van mezelf. Veel mensen hebben een bijdrage geleverd aan overige hoofdstukken van dit proefschrift, onderzoek doe je nooit alleen. Hen wil ik hier bedanken.

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Dr. Nobuhiro Yuki and Dr. Albert Lastovica: thank you for the pleasant collaboration, which contributed to two chapters in my thesis.

Mijn directe collega's, eerst arts-assistenten en nu AIOS Medische Microbiologie, maar daarom niet minder gezellig (al zijn de wekelijkse vrijdagmiddagborrels helaas wat in het slop geraakt). Velen van ons zitten in hetzelfde "opleiding-én-onderzoeks"-schuitje, waardoor het delen van de frustraties die dat vaak met zich meebrengt nooit een probleem was. Dank ook aan alle andere collega's van onze afdeling, voor de prettige sfeer en samenwerking. Het is geen straf om hier nog even te blijven. Jos, bedankt dat je mijn paranimf wilt zijn.

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Curriculum vitae

Curriculum vitae

Peggy Godschalk werd op 18 april 1975 geboren te Heerlen. In 1993 behaalde zij haar gymnasium diploma aan het Katholiek Gymnasium Rolduc te Kerkrade. In datzelfde jaar begon ze aan de studie Medische Biologie aan de Medische Faculteit van de Universiteit Utrecht. Na het behalen van de Propaedeuse in 1994 besloot ze om over te stappen op de studie Geneeskunde aan dezelfde faculteit. Van mei tot en met augustus 1997 deed ze haar wetenschappelijke stage getiteld "Construction and characterization of a DNA vaccine against infection with Semliki Forest Virus" aan het Eijkman-Winkler Instituut voor Medische Microbiologie in het UMC Utrecht, onder supervisie van Dr. Harm Snippe. Vervolgens verbleef ze enkele maanden in Malawi voor een onderzoek naar tuberculose ("Gender differences in relation to sputum submission and smear-positive pulmonary tuberculosis in Malawi") onder supervisie van Dr. Martin Boerée in het Queen Elisabeth Central Hospital te Blantyre. In januari 1998 behaalde zij haar doctoraal-examen Geneeskunde. Na het afleggen van het Artsexamen begon zij in september 2000 aan de afdeling Medische Microbiologie en Infectieziekten (MMIZ) van het Erasmus MC te Rotterdam aan haar promotieonderzoek (Prof. dr. Alex van Belkum). Van juni tot en met augustus 2001 bracht zij een werkbezoek aan het Institute for Biological Sciences te Ottawa, Canada. Tijdens dit bezoek werkte ze onder supervisie van Dr. Michel Gilbert en Dr. John Kelly aan het project "Detection of immunoreactive proteins of *Campylobacter jejuni* involved in the pathogenesis of the Guillain-Barré syndrome". In september 2002 begon ze bij de afdeling MMIZ als AGIKO aan de opleiding Medische Microbiologie (Prof. dr. Henri Verbrugh). Voor haar eerste internationale wetenschappelijke publicatie (Hoofdstuk 2 van dit proefschrift) ontving zij in 2005 de Kiemprijs van de Nederlandse Vereniging voor Microbiologie (NVvM). In september 2008 hoopt zij haar opleiding tot arts-microbioloog af te ronden.

Ze woont samen met haar vriend Daan Gunneweg en zoon Klaas in Rotterdam.

List of publications

List of publications

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