Eur J Clin Microbiol Infect Dis (2013) 32:207–226
DOI 10.1007/s10096-012-1733-4

ARTICLE

A novel link between *Campylobacter jejuni* bacteriophage defence, virulence and Guillain–Barré syndrome

R. Louwen · D. Horst-Kreft · A. G. de Boer · L. van der Graaf · G. de Knegt · M. Hamersma · A. P. Heikema · A. R. Timms · B. C. Jacobs · J. A. Wagenaar · H. P. Endtz · J. van der Oost · J. M. Wells · E. E. S. Nieuwenhuis · A. H. M. van Vliet · P. T. J. Willemsen · P. van Baarlen · A. van Belkum

Received: 3 August 2012 / Accepted: 13 August 2012 / Published online: 4 September 2012

Abstract Guillain–Barré syndrome (GBS) is a post-infectious disease in which the human peripheral nervous system is affected after infection by specific pathogenic bacteria, including *Campylobacter jejuni*. GBS is suggested to be provoked by molecular mimicry between sialylated lipooligosaccharide (LOS) structures on the cell envelope of these bacteria and ganglioside epitopes on the human peripheral nerves, resulting in autoimmune-driven nerve destruction. Earlier, the *C. jejuni* sialyltransferase (Cst-II) was found to be linked to GBS and demonstrated to be involved in the biosynthesis of the ganglioside-like LOS structures. Apart from a role in pathogenicity, we report here that Cst-II-generated ganglioside-like LOS structures confer efficient bacteriophage resistance in *C. jejuni*. By bioinformatic analysis, it is revealed that the presence of sialyltransferases in *C. jejuni* and other potential GBS-related pathogens correlated significantly with the apparent degeneration of an alternative anti-virus system: type II *C*lusters of *R*egularly *I*nterspaced *S*hort *P*alindromic *R*epeat and *as*sociated genes (CRISPR-Cas). Molecular analysis of the *C. jejuni* CRISPR-Cas system confirmed the bioinformatic investigation. CRISPR degeneration and mutations in the *cas* genes *cas2*, *cas1* and *csn1* were found to correlate with Cst-II

Electronic supplementary material The online version of this article (doi:10.1007/s10096-012-1733-4) contains supplementary material, which is available to authorized users.

R. Louwen (✉) · D. Horst-Kreft · G. de Knegt · M. Hamersma · A. P. Heikema · H. P. Endtz · A. van Belkum
Department of Medical Microbiology and Infectious Diseases, Erasmus MC, University Medical Center Rotterdam, 's-Gravendijkwal 230, 3015 CE, Rotterdam, The Netherlands
e-mail: r.louwen@erasmusmc.nl

A. G. de Boer · P. T. J. Willemsen
Central Veterinary Institute (CVI), Wageningen UR, Lelystad, The Netherlands

L. van der Graaf · J. A. Wagenaar
Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University Utrecht, Utrecht, The Netherlands

A. R. Timms
Division of Food Sciences, University of Nottingham, Nottingham, UK

B. C. Jacobs
Departments of Neurology and Immunology, Erasmus MC, Rotterdam, The Netherlands

J. van der Oost
Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands

J. M. Wells
Host–Microbe Interactomics, Animal Sciences, Wageningen University, Wageningen, The Netherlands

E. E. S. Nieuwenhuis · P. van Baarlen
Department of Pediatric Gastroenterology, Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands

A. H. M. van Vliet
Institute of Food Research, Norwich, UK

A. van Belkum
Microbiology Unit, bioMérieux, La Balme les Grottes, France

sialyltransferase presence ($p<0.0001$). Remarkably, type II CRISPR-Cas systems are mainly found in mammalian pathogens. To study the potential involvement of this system in pathogenicity, we inactivated the type II CRISPR-Cas marker gene *csn1*, which effectively reduced virulence in primarily *cst*-II-positive *C. jejuni* isolates. Our findings indicate a novel link between viral defence, virulence and GBS in a pathogenic bacterium.

Introduction

The bacterial pathogens *Campylobacter jejuni*, *Neisseria meningitidis*, *Haemophilus influenzae* and *Pasteurella multocida* are associated with severe disease or even death in mammals [1–4]. An important pathogenic feature of these pathogens is the utilisation of sialic acid, which is not only used as a carbon energy source, but also for sialylation of cell envelope-bound sugar structures [5]. The sialylated lipooligosaccharide (LOS) structures on the cell envelope of *C. jejuni*, *N. meningitidis*, *H. influenzae* and *P. multocida* contribute to human and animal serum resistance, immune evasion and increased virulence [6–13]. The transfer of sialic acid molecules to cell envelope-bound sugar structures in *C. jejuni* is catalysed by α2,3/α2,8-sialyltransferase Cst-II or α2,3-sialyltransferase Cst-III [14, 15], in *H. influenzae* by the Cst-II orthologous Lic3A/B [7] and in *P. multocida* by the α2,3-sialyltransferase PmST2 [16]. In *N. meningitidis*, the Lst α2,3-sialyltransferase and the SiaD α2,8- or α2,9-polysialyltransferase catalyse the transfer of sialic acid molecules to cell envelope-bound sugar structures on serotypes A, B and C species, respectively [17, 18]. Remarkably, the sialylated sugar structures in *C. jejuni*, *N. meningitidis* serotype B, *H. influenzae* and *P. multocida* all mimic sugar structures on the peripheral nerves of humans, called gangliosides [11, 16, 19, 20]. The ganglioside-like structures on these bacterial species are suspected to induce Guillain–Barré syndrome (GBS) in susceptible patients [21–25]. GBS is a severe subacute polyradiculoneuropathy with an annual incidence of 1.2–2.3 per 100,000 humans [26]. The current hypothesis is that a susceptible human host generates auto-antibodies that target both the bacterial ganglioside-like LOS structures and human peripheral nerve gangliosides, which triggers demyelination of the peripheral nerves, leading to paralysis [27, 28]. The gene encoding the *C. jejuni* Cst-II, which is required for the generation of ganglioside-like LOS structures GM1 and GD1, amongst others, is currently the only bacterial marker that has been correlated with GBS [29]. Noteworthy, the gene cluster harbouring *cst*-II is only observed in *C. jejuni* LOS loci classes A and B [30], and can be genetically exchanged between *C. jejuni* isolates [31, 32]. Three other common LOS loci in *C. jejuni* are the classes C, D and E, in which LOS class C harbours the sialyltransferase variant *cst*-III, enabling the expression of ganglioside-like structures other than GD1. In contrast, LOS classes D and E both lack sialyltransferases, making *C. jejuni* cells incapable in the expression of ganglioside-like structures [30].

Apart from the ability to express ganglioside-like structures on the bacterial cell envelope, *C. jejuni*, *N. meningitidis*, *H. influenzae* and *P. multocida* share a bacteriophage defence system, the type II *C*lusters of *R*egularly *I*nterspaced *S*hort *P*alindromic *R*epeat and *as*sociated genes (CRISPR-Cas), a system strongly reduced in size and complexity as compared to other CRISPR-Cas systems [33, 34]. The type II CRISPR-Cas is characterised by the *cas* gene *csn1*, which is absent in any of the other CRISPR-Cas subtypes [34]. CRISPR-Cas is an adaptive immune system, where the CRISPR spacers are transcribed, processed and used for viral defence by Cas protein-guided RNA interference [35, 36].

Remarkably, in mammals, ganglioside structures determine susceptibility to viruses [37–45]. Earlier, Levin predicted, in a mathematical model on CRISPR-Cas dynamics, that cell envelope-mediated bacteriophage resistance might affect CRISPR-Cas preservation [46]. Type II CRISPR-Cas as observed in the bacteria *C. jejuni*, *N. meningitidis*, *H. influenzae* and *P. multocida* could be the result of an independent bacteriophage defence system, e.g. cell envelope-bound ganglioside-like structures. This lead us to hypothesise that bacteriophage resistance in *C. jejuni* is mediated by variation in the expression of cell envelope-bound ganglioside-like structures, which, as a consequence, affects the preservation of CRISPR-Cas.

Materials and methods

Bacterial strains

Two hundred and thirty-nine clinical *C. jejuni* isolates (64 GBS-associated and 175 enteritis-inducing), two Δ*cst*-II mutants, four Δ*csn1* mutants, one *cst*-II complemented strain GB11 (C) and one *csn1*-supplemented 81176 isolate were used in this study. The specific protocols for the generation of the Δ*cst*-II mutants and complemented Δ*cst*-II mutant GB11 (C) strain can be found elsewhere [10, 30]. Generation of the four Δ*csn1* mutants and the *csn1*-supplemented isolate are described below. To minimise in vitro passaging, *C. jejuni* isolates were recovered from the original glycerol stock by culturing only once on Butzler agar plates (Becton Dickinson, Breda, The Netherlands). The Δ*cst*-II and Δ*csn1* mutants were then grown on blood agar plates, containing 7 % sheep blood (Becton Dickinson) supplemented with vancomycin (10 μg/ml) and

chloramphenicol (20 μg/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) for selection. The complemented GB11 (C) isolate and *csn1*-supplemented 81176 isolate were grown on blood agar plates, supplemented with 7 % sheep blood (Becton Dickinson) and erythromycin (0.01 μg/ml) (Sigma-Aldrich) for selection. Before use, a final passage on Columbia blood agar plates (Becton Dickinson) for wild types, Δ*cst*-II, Δ*csn1* mutants, Δ*cst*-IIΔ-complemented GB11 (C) and *csn1*-supplemented 81176 isolate were allowed for equal growth conditions. The LOS class for wild-type isolates was determined by polymerase chain reaction (PCR), using a previously described protocol [30]. All isolates were cultured under micro-aerophilic conditions at 37 °C, using anaerobic jars and an Anoxomat (Mart Microbiology B.V., Drachten, The Netherlands).

Phage susceptibility assay

Twenty-nine lytic *C. jejuni*-specific phages were used in this study. Thirteen phages were isolated from environmental sources; *Cje*001 to *Cje*009, *Cje*013, *Cje*015, *Cje*016 and *Cje*019, and 16 were purchased from the National Collection of Type Cultures (NCTC); NCTC666 and NCTC670 to NCTC684, for which distinct specificity profiles for *Campylobacter* were established [47]. In order to determine the susceptibility of *C. jejuni* to these phages and test the involvement of ganglioside-like structures, *cst*-II and *csn1* in bacteriophage defence, bacteriophage plaque assays were performed essentially as described by Connerton et al. [48]. Briefly, 6 ml of molten NZCYM overlay agar (0.7 %) (Becton Dickinson) was inoculated with 80 μl of a bacterial suspension (10^9 pfu/ml) of the *C. jejuni* isolates; GB2, GB3, GB4, GB5, GB11, GB15, GB17, GB19, GB22, GB23, GB29, 623, 624, 706, 9141, 9146, R65 and NCTC11168; GB2Δ*csn1*, GB11Δ*csn1*, GB19Δ*csn1* and NCTC11168Δ*csn1*; GB11Δ*cst*-II and GB19Δ*cst*-II and GB11 (C). Soft agar and bacterial suspensions were mixed and poured onto pre-warmed NZCYM agar (1.2 %) (Becton Dickinson) plates. After solidifying and drying the plates, each phage preparation containing approximately 10^7 pfu/ml was applied as a 10-μl spot to a prepared lawn and allowed to adsorb to the overlay agar. Plates were incubated for 24–48 h at 37 °C under micro-aerophilic conditions. Formed plaques were scored as: (1) not sensitive, (2) +indistinct lysis, (3) ++ partial lysis and (4) +++ complete lysis.

Analysis of CRISPR elements

The CRISPR element from individual isolates of which mass spectrometry data on LOS structures was available (27 GBS- and 58 enteritis-associated isolates) [49, 50] were amplified with the primer pair CRISPRFw and CRISPRRev (Table S1) (GenBank HQ378248–HQ378324). Amplification was performed in a 50-μl total volume, comprising 10 ng *Campylobacter* chromosomal DNA, 50 pmol of each primer, 20 mM dNTP (Fermentas, St. Leon-Rot, Germany), 5 μl of 10× Supertaq buffer (Sphaero Q, Gorinchem, The Netherlands) and 2 units of Supertaq (Sphaero Q). PCR assays were performed using a Biomed Thermal Cycler System 9700 (Applied Biosystems, Bleiswijk, The Netherlands) with a programme consisting of 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C. PCR products were checked for integrity on ethidium bromide-stained agarose gels and purified with the Zymoclean PCR kit (Zymo Research Corp., Orange, CA, USA). Purified DNA was sequenced using an ABI Prism 3700 automatic DNA sequencer according to the protocol of the manufacturer (Applied Biosystems). Sequence assembly and editing were performed with the SeqMan module of the DNASTAR package (DNASTAR Inc., Madison, WI, USA). CRISPR elements were identified, and individual spacers analysed, with the CRISPRFinder software http://crispr.u-psud.fr/Server/ [51].

Genome analysis

Comparisons between the presence of the CRISPR element and sialyltransferases occurred through the genome analysis of seven *C. jejuni*, 27 *N. meningitidis* and 24 *Haemophilus* spp. and a *P. multocida* genome sequences obtained from the GenBank database at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/. DNA and protein homology searches were performed using the BLAST executables.

Knock-out mutagenesis of *csn1*

The target gene *csn1* (Gene ID: 905809) and approximately 600 bp of flanking sequences were PCR-amplified and cloned into the pGem-Teasy vector (Promega Corp., Leiden, The Netherlands) [52]. Inverse PCR was used to introduce a *Bam*HI restriction site and a deletion of approximately 800 bp in the target genes [53]. Inverse PCR products were digested with *Bam*HI (Fermentas) and ligated to the *Bam*HI-digested chloramphenicol resistance (Cmr) cassette of pAV35 [53]. After sequencing, the resulting constructs were electroporated into different *C. jejuni* strains, and recombinants were selected on blood agar plates containing 7 % sheep blood (Becton Dickinson) and 20 μg/ml chloramphenicol (Sigma-Aldrich). Junction PCR, with primers up- and downstream of the area involved in the homologous recombination, and primers in the Cmr cassette, was performed to confirm double crossover events and to assess the orientation of the resistance cassette. For the primers, see Table S1.

Supplementation of gene *csn1* into 81176

For supplementation of the *csn1* gene, the pseudogene *Cj*0046 was used as the target gene. *Cj*0046 was previously shown to be useful for complementation in *C. jejuni* [54]. A construct was designed using plasmid pE46, useful for cloning the autologous *csn1* promoter and the *csn1* gene; the selection marker in these constructs was erythromycin. The vector uses a *Bsm*BI site for cloning. To clone the *csn1* gene into the pE46 vector, the forward primer BSMSBI-PROMFw and the reverse primer BSMBIRev were used (Table S1) to amplify the *csn1* gene with its own promoter. PCR products were checked for integrity on ethidium bromide agarose gels and purified with the Zymoclean PCR purification kit (Zymo Research Corp.). The pE46 vector and *csn1* PCR product were cloned. The resulting constructs were electroporated at 2.5 kV, 200 Ω, 25 μF into *Escherichia coli* Top10 cells (Invitrogen, Breda, The Netherlands) were resuspended in 37 °C pre-warmed SOB medium (Invitrogen) and allowed to recover by gentle shaking at 37 °C. After recovery, 100 μl was plated onto a Lysogeny broth (LB; Becton Dickinson) agar plate for selection containing 250 μg/ml erythromycin (Sigma-Aldrich). Colonies were screened by colony PCR for the presence of the *csn1* gene by using the primer pair Csn1FW1 and Csn1Rev1 (Table S1), covering approximately 450 bp at the beginning of the *csn1* gene, and the primer pair Csn2FW2 and CsnRev2 (Table S1), covering approximately 450 bp at the end of the *csn1* gene. Positive colonies were grown for plasmid DNA isolation (Fermentas) and subjected to different restriction digestions (*Bam*H1, *Hin*DIII and *Eco*RI) (Fermentas) to confirm the integrity of the constructs.

Electrotransformation of the *C. jejuni* isolates

On the day of electro-transformation of *C. jejuni*, the correct numbers of blood agar plates (with and without antibiotic) were put into a 37 °C incubator, to pre-warm. *C. jejuni* from the inoculated Columbian blood agar plate (Becton Dickinson) were harvested using LB supplemented with 1× non-essential amino acids (NEAA) (Invitrogen) and a spreader. *C. jejuni* was pelleted by centrifugation for 3 min at 14,000 rpm and resuspended with 1.5 ml of transformation buffer containing 272 mM sucrose (Sigma-Aldrich), 15 % (v/v) glycerol (Sigma-Aldrich) and 1× NEAA (Invitrogen), which was repeated one time. 0.5 ml of transformation buffer was used for resuspension without 1× NEAA (Invitrogen) and was aliquoted in 100-μl samples of the competent cells into 1.5-ml centrifuge tubes. The plasmid DNA (2 μg) to be transformed was suspended into the 100-μl sample of competent cells Δ*csn1* mutants or the 81176 isolate. The mixture was transferred to an electroporation cuvette and pulsed at 2.5 kV, 200 Ω, 25 μF. 1 ml of LB (Becton Dickinson) supplemented with 1× NEAA (Invitrogen) was added to the cuvette and mixed by pipetting to resuspend. 200 μl of the mix from the cuvette was plated and spread onto the surface of a pre-warmed labelled Columbian blood agar plates (Becton Dickinson). Plates were incubated for ~5 h at 37 °C under micro-aerophilic conditions. The cells were recovered from the plate using 2 ml of LB supplemented with 1× NEAA (Invitrogen) and a spreader. The suspension was spread on the surface of a new pre-warmed blood agar plate containing 0.2 μg/ml chloramphenicol (Sigma-Aldrich) for selection of the knock-out mutants and erythromycin (Sigma-Aldrich) was used with a concentration of 0.01 μg/ml to screen for supplemented *csn1* 81176 clones or GB11 (C) complemented clones. Plates were incubated at 37 °C under micro-aerophilic conditions for 2–5 days, until colonies are visible. Colonies resistant to chloramphenicol or erythromycin were passaged five times on new plates to generate stable clones. From these stable clones, genomic DNA was isolated using QIAamp® DNA tissue stool kit (Qiagen) for further analysis. A PCR assay was used to test for the correctness of the supplementation assay. For supplementation, we used the primer pairs presented in Table S1 to confirm that the supplemented strains had the gene inserted in the sense orientation.

Phage sensitivity in broth medium

Phage CP28 was used to determine *C. jejuni* resistance to this phage in the presence or absence of the *csn1* gene in a liquid environment. Wild-type isolates (GB2, GB11, GB19 and NCTC11168) and their Δ*csn1* mutants (GB2Δ*csn1*, GB11Δ*csn1*, GB19Δ*csn1* and NCTC11168Δ*csn1*) were grown overnight at micro-aerophilic conditions at 37 °C. After overnight growth, a *C. jejuni* suspension with an optical density (OD) of 0.5–0.6 was prepared in Mueller Hinton broth (Becton Dickinson), in which a 20-μl phage suspension was mixed containing $\log_{10}$ 4 to 5 pfu/ml of the CP28 phage. The OD_{600nm} of the *C. jejuni* and phage suspension was followed over time to analyse for resistance (opaque suspension: growing bacteria) or susceptibility (cleared suspension: lysis) to the phage CP28. Non-infected *C. jejuni* suspensions of a wild type and a Δ*csn1* mutant were included in the assays to control for growth and lysis.

PCR-RFLP analysis

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed on 26 GBS-inducing and 126 enteritis-inducing (Dutch) isolates that were matched on GBS and enteritis patient age and gender. To amplify *cas2* (Gene ID: 905810) and *cas1* (Gene ID: 905808), the primers presented in Table S1 were used.

PCR assays were performed using a Biomed Thermal Cycler System 9700 (Applied Biosystems) with a programme consisting of 35× of the following cycling protocol: 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C. Per reaction, approximately 50 ng of template genomic 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 dNTPs (Fermentas) and 0.2 units of SuperTaq polymerase (Sphaero Q). For restriction analysis, PCR products were subjected to overnight incubation at 65 °C with *Tsp*509I (Fermentas). Length determination of the PCR products and their *Tsp*509I RFLP products was performed by using electrophoresis of fragments in 1.5 % and 3 % agarose gels, respectively. Single-band differences between the PCR-RFLP types led to the designation of novel RFLP types.

PCR and sequencing of the *cas* gene *csn1*

The full-length *csn1* gene (Gene ID: 905809) of 27 *C. jejuni* isolates was amplified with the primer pair shown in Table S1. PCR assays were performed using a Biomed Thermal Cycler System 9700 (Applied Biosystems) with a programme consisting of 35 cycles of 1 min at 95 °C, 2 min at 55 °C and 3.5 min at 72 °C. Per reaction, approximately 50 ng of template genomic DNA was used in a buffer 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 dNTPs (Fermentas) and 0.2 units of SuperTaq polymerase (Sphaero Q) and a proof-reading enzyme Pfu polymerase (Fermentas). Length determination of the PCR products was performed by electrophoresis using 0.8 % agarose gels (Sphaero Q). PCR products were purified with the Zymoclean PCR kit (Zymo Research Corp.). For DNA sequencing, the fluorescence-labelled dideoxy technology was used according to the protocol of the manufacturer (Applied Biosystems). To sequence the entire *csn1* PCR product, additional forward and reverse primers had to be designed, as given in Table S1. Non-incorporated dye terminators were removed with the multiscreen assay system (Millipore). The 27 sequences were aligned using Sci Ed Central (Scientific & Educational Software, Cary, NC, USA), to which eight genome reference sequences of three GBS-associated isolates, HB9313, 260.94 and ICDCCJ07001, and five enteritis-inducing isolates, 11168, 81116, RM1221, 8425 and CG8486, were added.

PCR and sequence analysis of GBS-associating polymorphisms

The *csn1* gene (Gene ID: 905809) of 239 *C. jejuni* isolates (64 GBS-associated and 175 enteritis-inducing isolates were analysed for the presence of the observed triplet CAA and GAA polymorphism with the primer sets presented in Table S1. The CAA and GAA analysis was also performed on 26 GBS-associated and 120 enteritis-inducing (Dutch) isolates that were matched on GBS and enteritis patient age and gender. Single-nucleotide polymorphism (SNP) analysis at 2,058 bp, 2,073 bp and 2,092 bp occurred by PCR amplification and sequencing of the *csn1* gene at 1,891–2,433 bp from the start site using 26 GBS- and 69 enteritis-inducing isolates and the primer set presented in Table S1. PCR assays were performed using a Biomed Thermal Cycler System 9700 (Applied Biosystems) with a programme consisting of 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C. Per reaction, approximately 50 ng of template genomic 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 dNTPs (Fermentas) and 0.2 units of SuperTaq polymerase (Sphaero Q). Length determination of the PCR products was performed by electrophoresis using 1.5 % agarose gels (Sphaero Q) and sequencing occurred as mentioned above.

Molecular clock BEAST analysis

Molecular clock phylogenetic signals in the base substitutions in the *C. jejuni* CRISPR elements and *csn1 cas* gene were analysed using BEAST v.1.5beta3 [55]. BEAST input XML files were generated using BEAUti v.1.5beta2. The BEAST output was examined using Tracer 1.4 [55]. This program calculates frequency distributions and outputs statistical parameters and their support through the effective sample size (ESS) index. The ESS index of a parameter reflects the reliability of distributions, such that a small ESS index indicates that the estimate of the posterior distribution of that parameter will be poor. Well-supported parameters have ESS index>200; poorly supported parameters have 100<ESS index<200; parameters with ESS index<100 should not be used. BEAST analysis was used to analyse whether *cst*-II-positive isolates evolved later than the *cst*-II-negative isolates, by exploring the time to the most recent common ancestor (TMRCA) using the CRISPR element or *cas* gene *csn1* sequences.

Adhesion and invasion assays

Human intestinal epithelial Caco-2 and HT-29 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen) and 1 % NEAA (Invitrogen), as previously described [10]. Adhesion and invasion by *C. jejuni* was determined by growing the intestinal epithelial cells (Caco-2) in a multi-well culture plate (six-well plate, Greiner Bio-One, Alphen a/d Rijn, The Netherlands) to confluence until dome-forming occurred, an indication for

differentiation [56]. The adhesion and invasion assays were further performed as previously described [10].

Translocation of *C. jejuni* in a Transwell system

Caco-2 cells were seeded onto Transwell filters at 4×10^5 cells/filter (5-μm pore size, 1.13 cm^2; Costar, Corning Inc., Corning, NY, USA) and allowed to differentiate and form tight junctions for 19 days. After 7–10 days of culture, the transepithelial electrical resistance (TEER) was>1,000 Ω/cm^2, indicating the presence of an intact epithelial monolayer. *C. jejuni* isolates were added at a multiplicity of infection (MOI) of 10 to the apical surface of the Transwell filter at day 19. After 24, 48 and 72 h, 10-μl samples were taken from the apical and basolateral surface. Serial dilutions (1:100) were made and plated on Columbia blood agar plates (Becton Dickinson). Plates were incubated for 24 h at 37 °C in an anaerobic jar (Mart) under micro-aerobic conditions. Colonies were counted and the CFU/ml was calculated.

Swarming analysis

C. jejuni suspensions were prepared with an OD_{600nm} of 1 and a semi-solid agar plate (MH broth plus 0.4 % agar) was inoculated with 1 μl of bacterial culture by stabbing. Plates were grown at 37 °C under micro-aerophilic conditions and the resulting circle diameters exemplifying bacterial swarming were measured after incubation for 24 h. This swarming analysis and diameter motility zone measurements were performed three times for wild-type and Δ*csn1* strains, respectively.

Cytotoxicity assay

The post-experimental lactate dehydrogenase (LDH) content in the supernatant of untreated HT-29 controls and of *C. jejuni* GB2, GB11, GB19, NCTC11168 and their Δ*csn1* variants overnight-treated HT-29 intestinal epithelial cells was determined using LDH release as an indicator of membrane damage. LDH release was measured using the Cell Toxicity Colorimetric Assay Kit (Sigma-Aldrich). Incubation was performed in DMEM medium without phenol red (Invitrogen) and only containing 1× NEAA (Lonza). The total LDH content of the residual cells was measured by subtracting the results from the treated cell samples at an OD_{490nm} from the untreated controls. The experiment was repeated three times.

Recombinant Csn1 expression, purification and polyclonal antibody production

E. coli DH5-α (Invitrogen) was used for sub-cloning. *E. coli* BL21 (DE3) (Invitrogen) was used for the heterologous expression of Csn1. pET11c was the protein expression plasmid (Invitrogen). Restriction enzymes *Nhe*1 and *Bam*H1 and T4 DNA ligase were purchased from Fermentas. The forward primer pET11c1523Fw and the reverse primer pET11c1523Rev containing a *Bam*H1 restriction site, stop codon and six histidines encoding triplets presented in Table S1 were used to amplify the *csn1* gene. pET11c (Invitrogen) and the purified *csn1* PCR product was digested with *Nhe*1 and *Bam*H1 (Fermentas) overnight at 37 °C, purified again and ligated using T4 DNA ligase (Fermentas) overnight at 16 °C. The pET11c (Invitrogen) plasmid containing the *csn1* gene was transformed into the *E. coli* strain BL21 (DE3) (Invitrogen) for expression. An Ni-NTA Fast Start Kit was used for Csn1 purification. Expression and purification occurred according to the protocol of the manufacturer (Qiagen). The Csn1 protein was then subjected to 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified proteins were dialysed against HBSS (pH 7.0) (Invitrogen) with a descending guanidine HCl isothiocyanate gradient from 2 M to 0 M, to prevent precipitation of the Csn1 protein during dialysis. Protein concentrations were measured using NanoDrop (Ocimum Biosolutions, IJsselstein, The Netherlands). The protein solution was diluted to one with a final concentration of 1 mg/ml and sent to Genway Biotech Inc. (San Diego, CA, USA) for polyclonal antibody production in rabbits. The pre-immune serum of these rabbits was used as a negative control in the Csn1 detection experiments.

SDS-PAGE and Western blot

Fresh overnight *C. jejuni* cultures of GB2, GB11, GB19, 11168 and their Δ*csn1* mutant strains were harvested in ice-cold phosphate-buffered saline (PBS) and lysed using lysis Matrix B (Sanbio, Uden, The Netherlands) in the Fastprep FP120 machine (Thermo Savant, Holbrook, NY, USA) for 25 s at speed 6. Lysates were centrifuged at 14,000 rpm for 5 min at 4 °C and were treated or not with proteinase K (1 mg/ml) at 65 °C for 2 h. In order to analyse LOS and culture supernatants for the presence of LOS, TCA (Sigma-Aldrich) precipitation was used to concentrate the LOS that was present in the culture supernatants. Precipitated supernatants, lysed strains and proteinase K-treated lysates were run in a 12 % SDS-PAGE gel. Gels were blotted onto a polyvinylidene fluoride (PVDF) nitrocellulose membrane. Membranes were blocked with PBS containing 5 % non-fat dry milk (BioRad) and 0.01 % Tween-20 (Sigma-Aldrich) for 1 h at room temperature. Sera from GBS patients from which GB2, GB11 and GB19 were isolated were used at a dilution of 1:2,500 and incubated for 2 h at room temperature. Goat anti-human total IgG labelled with alkaline phosphatase (Promega, Leiden, The Netherlands) at a dilution of

1:5,000 was used as a secondary antibody. As a loading control, peroxidase-conjugated cholera toxin (Sigma-Aldrich) was used at a concentration of 1 μg/ml to verify that equal amounts of LOS were loaded on the gel initially. The detection of Csn1 was done with the polyclonal antibodies generated in rabbits against Csn1 1:1,000 and a secondary antibody anti-rabbit IgG alkalic phosphatase labelled 1:1,000. Visualisation occurred with NBT/BCIP solution (Sigma-Aldrich).

Statistics

Phage sensitivity between ganglioside mimic-positive and ganglioside mimic-negative, wild-type (GB11 and GB19) and their Δ*cst*-II mutants was analysed on significant resistance or susceptibility differences using a contingency table and a Chi-square test. PCR-RFLP data from the *cas2* and *cas1* were tested for associations with the LOS locus (presence or absence of *cst*-II) or disease outcome (GBS or enteritis) using Fisher's exact probability or Chi-square tests, were appropriate. Statistical analysis on the observed PCR-RFLP types was performed using the Statistical Package for the Social Sciences software version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). *csn1* polymorphisms were analysed using VassarStats contingency table software (http://vassarstats.net/). A Fisher's exact probability test or Chi-square test was selected, where appropriate, to analyse the detected polymorphisms for significance. A paired *t*-test using Instat software (version 2.05a; GraphPad Software, San Diego, CA, USA) was used to verify differences in adhesion, invasion, translocation and cytotoxicity between wild-type and Δ*csn1* mutants. Log transformation was used to normalise variances. A two-tailed value with $p<0.05$ indicated statistical significance.

Results

C. jejuni ganglioside-like structures provide effective bacteriophage resistance

Assessment of the bacteriophage resistance of 11 GBS- and seven enteritis-derived isolates revealed that isolates expressing ganglioside-like structures GM1 and GD1 were significantly more resistant to 29 different lytic *C. jejuni*-specific bacteriophages than isolates lacking such ganglioside-like structures (Table S2) (81 and 64 versus 94 and 282; Chi-square test, $p<0.0001$). In more detail, the bacteriophages NCTC12669–674 and NCTC12678–680, which use cell envelope structures to establish an infection [47], were not able to infect strains carrying the ganglioside-like structures GM1 and GD1 (Table 1). In contrast, isolates that lack the GM1 and GD1 ganglioside-like structure were significantly more susceptible to the bacteriophages NCTC12669–674 and NCTC12678–680 (Table 1) (42 and 3 versus 38 and 79; Chi-square test, $p<0.0001$).

The bacteriophages *Cje*001–009, *Cje*13, *Cje*15, *Cje*16 and *Cje*19 are new isolated untyped Dutch *C. jejuni* bacteriophages, for which the *C. jejuni* receptor to establish an infection is not yet known. The *C. jejuni* susceptibility profile for bacteriophages *Cje*001–005, *Cje*013 and *Cje*015 was similar to the bacteriophages NCTC12669–674 and NCTC12679–680, with the exception the NCTC12678 bacteriophage (Table S2). Remarkably, the *C. jejuni* isolates with a class E LOS locus (623, 624, 9141, 9146 and GB4), were also resistant to bacteriophages NCTC12669–674 and NCTC12678-680 (Table 1). However, comparing the level of bacteriophage resistance between class E LOS locus isolates (623, 624, 9141, 9146 and GB4) and class A LOS locus GM1- and GD1-expressing isolates (GB2, GB3, GB11, GB19 and GB22) showed that GM1 and GD1 expression provides superior resistance to the bacteriophages NCTC12669–674 and NCTC12678–680 (Table 1) (42 and 3 versus 32 and 13; Chi-square test, $p=0.0058$).

The involvement of the ganglioside-like structures GM1 and GD1 in bacteriophage resistance was further confirmed by inactivation of the sialyltransferase gene *cst*-II in the GM1- and GD1-expressing *C. jejuni* isolates GB11 and GB19. We found that the two Δ*cst*-II mutants (GB11Δ*cst*-II and GB19Δ*cst*-II) were susceptible to twice as many of the 29 lytic *C. jejuni*-specific bacteriophages tested, compared to their wild-type parent isolates (GB11Δ*cst*-II susceptible for 27/29 vs. GB11 susceptible for 14/29; Chi-square test, $p=0.0002$; GB19Δ*cst*-II susceptible for 26/29 vs. GB19 susceptible for 13/29; Chi-square test, $p=0.0003$) (Table S3). Genetic *cst*-II complementation of the GB11Δ*cst*-II mutant partially restored bacteriophage resistance (Table S3). We found, after further investigation, that the intermediate resistance level as observed between the complemented Δ*cst*-II mutant and wild-type isolate correlated well with restored expression of GM1 but not GD1 (Table S3). *cst*-II complementation also established that the new Dutch *Cje* bacteriophages do not infect *C. jejuni* isolates with GM1 structures only (Table S3). GM1 expression on the complemented Δ*cst*-II mutant GB11 (C) did, thus, reduce viral susceptibility to the NCTC12669–674 and NCTC12678–680 bacteriophages, but due to the lack of GD1 expression, the GB11 (C) remained susceptible, although to a lesser extent.

Presence of bacterial species-specific sialyltransferases affects the preservation of CRISPR-Cas

An earlier study on the dynamics of CRISPR-Cas evolution suggested that the development of alternative bacteriophage resistance systems (e.g. cell envelope modification) could

Table 1 Ganglioside-like structures GM1 and GD1 provide efficient protection against NCTC bacteriophages

Isolate[a]/phage[b]	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	LOS class[c]	*cst*-II[d]	LOS structure[e]
GB2	−	−	−	−	−	−	+++	+++	+++	−	−	−	−	+++	+++	+++	A	+	GM1/GD1
GB11	−	−	−	−	−	−	+++	+++	++	+	−	−	+++	+++	+++	+++	A	+	GM1/GD1
GB19	−	−	−	−	−	−	+++	+++	+++	−	−	−	+++	+++	+++	+++	A	+	GM1/GD1
GB22	−	−	−	−	−	−	++	++	+	+	−	−	++	+	+	++	A	+	GM1/GD1
GB3	−	−	−	−	−	−	++	++	+	+	−	−	++	−	−	++	A	+	GM1/GD1
GB23	+++	+++	+++	+++	+++	+++	++	+++	+++	++	+	++	++	+	+	++	A	+[†]	GM2
GB5	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+	+	+	++	B	+[‡]	None
GB17	++	+	+	+	++	++	+++	++	+	++	+	+	++	+	+	++	B	+[†]	GA1/GM1/GD1
GB29	+++	+++	+++	+++	+++	+++	++	++	++	+	++	+	+	+	++	++	C	−	None
11168	−	+	+	++	+++	+++	−	−	−	+	+++	+++	−	−	+	+	C	−	GM1/GM2
706	−	++	++	+	++	++	+++	+++	++	+	+	+	+++	++	+	+++	D	−	None
GB15	−	++	−	−	+	+	++	++	++	+	+	−	++	++	+	++	D	−	None
R65	++	+++	++	++	++	+	++	++	+	++	+++	+	++	+	+	++	D	−	None
623	−	−	−	−	−	−	+++	+	+	−	−	−	++	+	+	++	E	−	None
624	−	−	−	−	−	−	+++	++	++	−	−	−	++	+	+	++	E	−	None
9146	++	−	++	++	+	−	++	+++	++	++	−	+	+	+	+	++	E	−	None
9141	+++	−	+++	+++	−	−	++	+++	++	+++	−	++	++	+	+	++	E	−	None
GB4	−	++	−	−	−	+	+++	+++	++	−	−	−	+	+	+	++	E	−	None

[a] Isolates used in the bacteriophage plaque assay GB are GBS-associated isolates; R or number only are enteritis-associated isolates (shown vertically)

[b] Shows the used 16 NCTC bacteriophages 12669–684 described previously (shown horizontally) [47]. Bacteriophages 669–674 and 678–680 target the cell envelope for bacterial entry, whereas bacteriophages 675–677 and 682–684 target the flagella [47]

[c] LOS class determined by PCR as described by Godschalk et al. [30]

[d] *cst*-II presence determined by PCR as described by Godschalk et al. [30]

[e] LOS structure determined by mass spectrometry as described previously [49, 50]; GA1 means asialoganglioside-like LOS; none means the absence of ganglioside-like LOS. Experiments were repeated four times and the average susceptibility is shown

−= no lysis; + = indistinct lysis; ++ = partial lysis; +++ = complete lysis

[†] Mutations in *cst*-II or *cgtB* gene influencing ganglioside-like structure expression

[‡] This isolate lacks a functional *cgtB* gene needed for the generation of a sialic acid acceptor for Cst-II, which disables the expression of GM1 and GD1 ganglioside-like structures in this isolate [49]

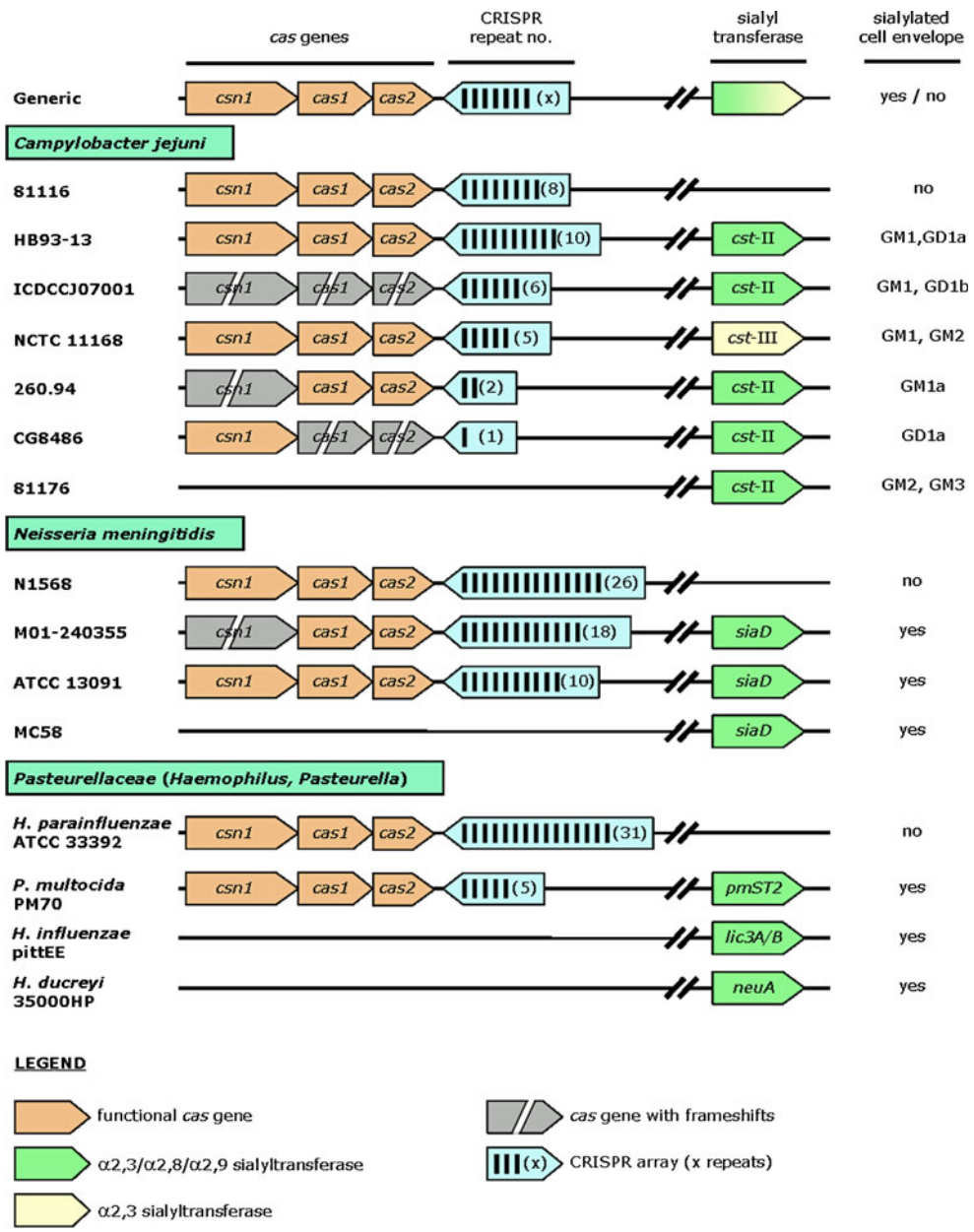
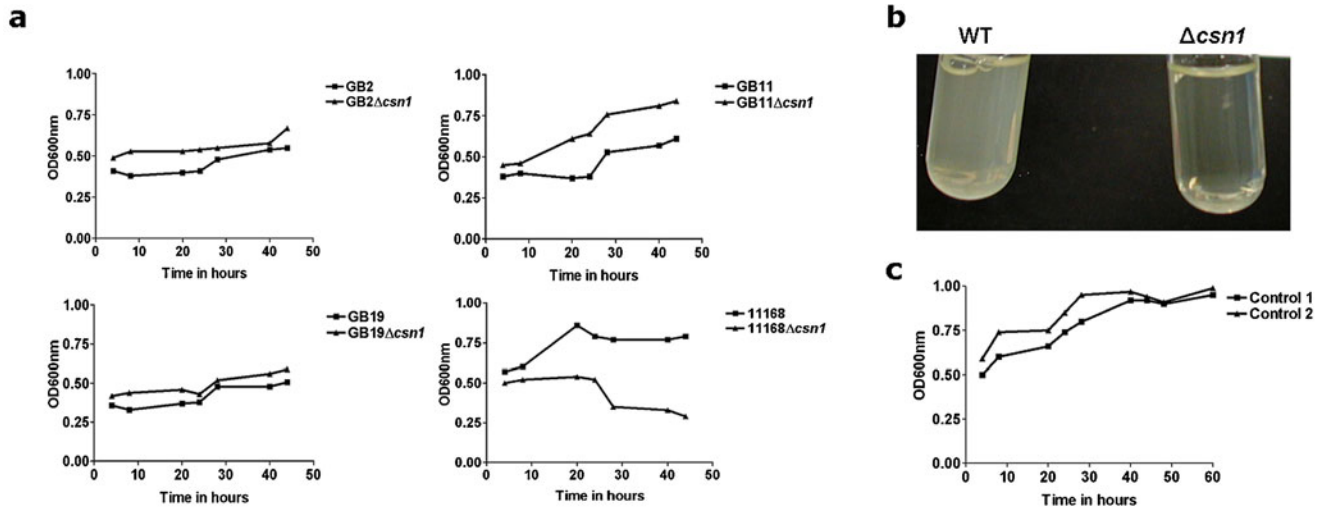


Fig. 1 Sialyltransferase presence associates with a reduced or absent CRISPR-Cas system. *C. jejuni*, *N. meningitis*, *Haemophilus* spp. and *P. multocida* show a remarkable similarity with their CRISPR-Cas system. They all harbour a reduced type II CRISPR-Cas subtype and a species-specific sialyltransferase for cell envelope sialylation. Bioinformatic analysis of seven *C. jejuni* reference isolates revealed that the presence of the sialyltransferase *cst*-II correlated in four of the five *cst*-II-positive isolates (ICDCCJ07001, 260.94, CG8486, 81176) with a reduced, frameshifts-harbouring (ICDCCJ07001, 260.94, CG8486) or absent type II CRISPR-Cas system (81176). Bioinformatic analysis on 27 *N. meningitidis* reference isolates revealed that three harboured a N1568 CRISPR-Cas like system, two were comparable with the M01-240355 CRISPR-Cas system and 18 were similar to MC58, an isolate that lacks a CRISPR-Cas system. In *N. meningitidis*, we established a link between the presence of a sialyltransferase, in *N. meningitidis siaD*, and a reduced, frameshifts-harbouring or absent type II CRISPR-Cas system as well. For four *N. meningitidis*, we observed an aberrant pattern. Bioinformatic analysis of 21 *H. influenzae*, three *H. parainfluenzae* and a *P. multocida* reference isolate also revealed a link between the presence of a sialyltransferase and a reduced or absent type II CRISPR-Cas system. The 21 *H. influenzae* isolates harboured the sialyltransferase *lic3A/B*, a *C. jejuni cst*-II homologue, but all lacked a type II CRISPR-Cas system. The three *H. parainfluenzae* isolates contain a complete type II CRISPR-Cas system, but lack a sialyltransferase. *P. multocida* harbours a reduced CRISPR element, which associates with the presence of the sialyltransferase *PmST2*

lower the evolutionary pressure on the CRISPR-Cas system [46]. Hence, we investigated whether the presence of a *cst*-II like sialyltransferase was linked with specific changes in the type II CRISPR-Cas system. Bioinformatic screening of seven *C. jejuni* reference isolates revealed a potential link between Cst-II-mediated ganglioside-like structures GM1



d

Strain/phage	*Cje*001	*Cje*002	*Cje*003	*Cje*004	*Cje*005	*Cje*006	*Cje*007	*Cje*008	*Cje*009	*Cje*013	*Cje*015	*Cje*016	*Cje*019	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684
GB2	-	-	-	-	-	+++	+++	++	+++	-	-	+++	++	-	-	-	-	-	-	+++	+++	+++	-	-	-	-	+++	+++	+++
GB2Δ*csn1*	-	-	-	-	-	+++	++	+	+++	-	-	++	++	-	-	-	-	-	-	+++	+++	+++	-	-	-	+++	+++	+++	+++
GB11	-	-	-	-	-	+++	+++	+++	+++	-	-	++	+++	-	-	-	-	-	-	+++	+++	++	+	-	-	+++	+++	+++	+++
GB11Δ*csn1*	-	-	-	-	-	+	++	+	++	-	-	++	++	-	-	-	-	-	-	+++	+++	++	-	-	-	+	+	++	++
GB19	-	-	-	-	-	+++	++	+	++	-	-	+++	+	-	-	-	-	-	-	+++	+++	+++	-	-	-	+++	+++	+++	+++
GB19Δ*csn1*	-	-	-	-	-	-	++	+	++	-	-	++	++	-	-	-	-	-	-	+++	+++	++	+	-	-	+	+++	+++	+++
11168	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+	+	++	+++	+++	-	-	-	+	+++	+++	-	-	+	+
11168Δ*csn1*	+	+	+	+	+	+	-	-	+	+	+	-	-	-	+	+	+++	+++	+++	+	-	-	+++	+++	+++	-	-	++	++

Fig. 2 Role of CRISPR-Cas and ganglioside-like LOS in the phage resistance of *C. jejuni*. **a** Phage lysis was measured at an optical density (OD) of 600 nm. Time is shown in hours. Experiments were repeated three times. The mean is shown. **b** WT is the wild-type isolate NCTC11168 and Δ*csn1* represents the NCTC11168Δ*csn1* mutant. Opaque bacterial suspension indicates no significant lysis and a clear suspension indicates significant lysis. **c** A wild-type isolate and a Δ*csn1* mutant not exposed to the phage CP28 revealed only growth overtime. **d** The role of *csn1* in phage susceptibility was further analysed by subjecting the GBS isolates GB2, GB11 and GB19, the enteritis isolate NCTC11168 and their Δ*csn1* mutants to 29 different lytic *C. jejuni*-specific phages; assay was repeated four times. The mean results are shown. The different degradations of lysis are marked by: − = no lysis; + = indistinct lysis; ++ = partial lysis; +++ = complete lysis. Worthy of mention, the GBS-inducing isolates GB2 and GB19 both harbour only one CRISPR spacer, whereas GB11 harbours no CRISPR spacers (Table S4), whereas the enteritis isolate NCTC11168 contains four CRISPR spacers in its CRISPR element (Fig. 1). Successful knock-out mutagenesis of *csn1* is shown with a Western Blot (Fig. S5)

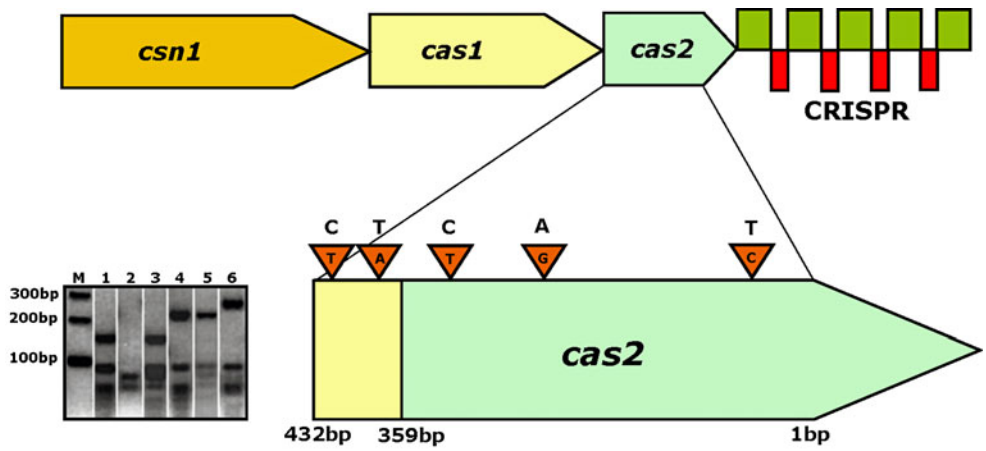
and/or GD1 and a degenerated *C. jejuni* CRISPR-Cas system. We observed that strains with a *cst*-II gene harboured mutations and frameshifts (CG8486, taxid: 398000 and ICDCCJ07001, taxid: 757425, as presented in the NCBI database) in the *cas* genes or contained short (1–3 repeats) or no CRISPR element (Fig. 1). This observation was supported by the CRISPR analysis of 40 *jejuni* clinical isolates, for which Cst-II-mediated ganglioside-like structure expression was established [49, 50]. We found that the expression of ganglioside-like structures GM1 and/or GD1 correlated strongly with a short (1–3 repeats) or an absent CRISPR element (two-tailed Fisher's exact test, 20 and 7 versus 2 and 11; p=0.0007) (Table S4). Further bioinformatic screening of 27 *N. meningitidis*, 24 *Haemophilus* spp. and one *P. multocida* genome sequences established that this finding was not *C. jejuni*-specific, as the presence of sialyltransferases in these bacterial species also correlated with a degenerated CRISPR-Cas system (Fig. 1).

CRISPR-Cas plays only a minor role in bacteriophage defence in *C. jejuni* isolates expressing GM1 and GD1 epitopes

To investigate the functionality of the *C. jejuni* CRISPR-Cas system in the presence or absence of GM1 and GD1 structures in bacteriophage defence, the *cas* gene *csn1* was inactivated in three *cst*-II-positive isolates (GB2, GB11 and GB19) and in one *cst*-II-negative isolate (NCTC11168). NCTC11168 was chosen because this isolate, based on the gene content, is very similar to GB11. An important difference between GB11 and the NCTC11168 isolate is that the latter misses the LOS loci harbouring the *cst*-II gene [31]. Inactivation of the *cas* gene *csn1* in the NCTC11168 isolate resulted in bacterial lysis visualised by a 50 % drop of the OD_{600nm} when the 11168Δ*csn1* mutant and bacteriophage CP28 were co-cultured together in Mueller Hinton broth (Fig. 2). In contrast, the CP28 bacteriophage did not affect the three *cst*-II-positive *C. jejuni* isolates expressing GM1

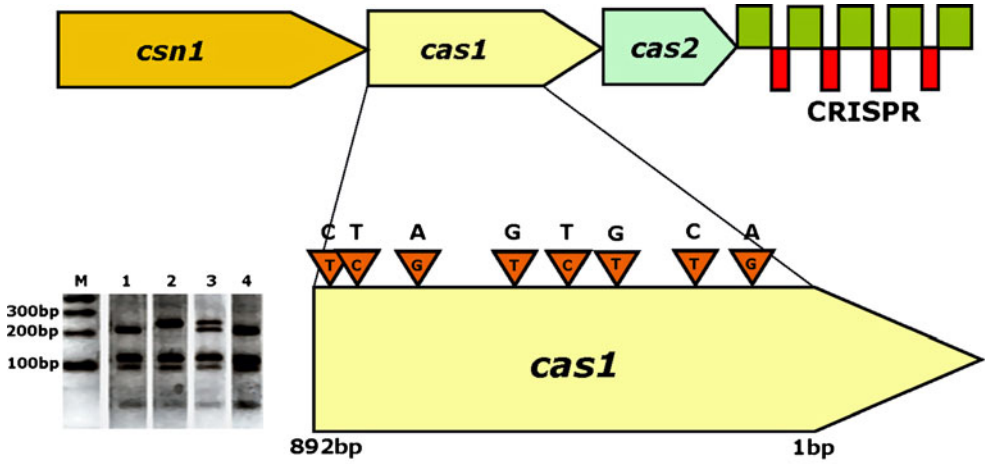
Spacer [a]	NCBI alignment CRISPRFinder [b]	Percentage [c]	Alignment *C. jejuni* genome [d]		
AE	pVir plasmid present in 81176 *C. jejuni* strain	96%	1 4556	TGATTTATTAGTAACCCATCAAGTTGGCTT TGATTTATTAGTAAC**A**CATCAAGTTGGCTT	30 4586
AK	PHAGEO3 present in RM1221 *C. jejuni* strain	100%	1 1348527	AGAATACCTGAAGGAAATTATAATTTAAGA AGAATACCTGAAGGAAATTATAATTTAAGA	30 1348497
BA	PHAGEO2 present in RM1221 *C. jejuni* strain	100%	1 535673	CCATCAAGTCGTGCAATTTTAATACACTGG CCATCAAGTCGTGCAATTTTAATACACTGG	30 535703
BW	*C. jejuni* phage CP81	100%	1 54163	ATGATGAAAGTTCTAATTATGTTAAAGTTT ATGATGAAAGTTCTAATTATGTTAAAGTTT	30 54193
BW	*C. jejuni* phage NCTC12673	96%	1 120610	ATGATGAAAGTTCTAATTATGTTAAAGTTT ATGATGA**G**AGTTCTAATTATGTTAAAGTTT	30 120580
CD	Phage regulatory protein Rha *C. doylei* strain	77%	1 974570	AATAATGATATCGAAGTTATTAATCTAATTG AATAATGATATCG**T**AG**A**TATTAATCT	31 974544
DL	PHAGEO3 present in RM1221 *C. jejuni* strain	100%	1 1348395	GCAATTTTAATACACTGGGGAAACACTTAT GCAATTTTAATACACTGGGGAAACACTTAT	30 1348365

Fig. 3 CRISPR spacers show 100 % identity with *C. jejuni* (pro-)phages. CRISPR spacer sequences did not show any identity with the exchangeable *cst*-II gene (GenBank accession number AY422197), but did reveal 100 % identity with *C. jejuni* (pro-)phage sequences and a virulence plasmid pVir, where: **a** represents the spacer column showing the alphabetically numbered spacers that revealed significant identity; **b** shows the target to which this specific spacer is aligned; **c** reveals the amount of identity in % that the positive spacer had with its target sequence; **d** alignment *C. jejuni* genome reveals the spacer sequences with a *C. jejuni* genome sequence corresponding to a (pro-)phage sequence found in *C. jejuni* RM1221. In **bold** and underlined are the observed mutations between spacer and bacteriophage sequences, respectively



RFLP Type →	1	2	3	4	5	6	Δ
GBS +	14	0	6	1	3	1	1
GBS -	48	1	51	5	11	1	3
cst-II +	34	0	16	1	7	1	3
cst-II -	28	1	41	5	7	1	1

Fig. 4 *cas2* gene polymorphisms associate with *cst*-II sialyltransferase gene presence. Bioinformatic analysis of *cas2* (Gene ID: 905810; 432 bp) using the *C. jejuni* genome reference sequences revealed DNA polymorphisms that enabled or disabled digestion with the restriction enzyme *Tsp*509I. In the *orange triangle*, the nucleotides are shown that were observed to be mutated in *cas2*. Above the orange triangles, the nucleotides are shown corresponding to the observed mutation that enabled or disabled *Tsp*509I digestion at the nucleotide recognition site AATT. For *cas2*, the mutations were found at positions 73, 246, 313, 378 and 428 bp from the gene start site. We also observed that some isolates lacked 73 bp, from 359 bp to 432 bp, at the end of *cas2* (*yellow block*). An overview is given of the six different PCR-RFLP types detected for the *cas2* gene as visualised on a 3 % agarose gel. *M* represents the marker in bp for which the 100-, 200- and 300-bp marker recognition sites are shown. *Numbers 1 to 6* correspond to the different detected PCR-RFLP. PCR-RFLP type Δ corresponds to a negative PCR reaction for *cas2*. PCR-RFLP type 1 correlates with the presence of the *cst*-II gene locus (Fisher's exact probability test, $p=0.011$), whereas PCR-RFLP type 3 correlates with the absence of the *cst*-II gene locus (Fisher's exact probability test, $p=0.0060$). PCR-RFLP type 3 strongly associates with LOS class E, a LOS class that lacks *cst*-II (Fisher's exact probability test, $p<0.0001$)



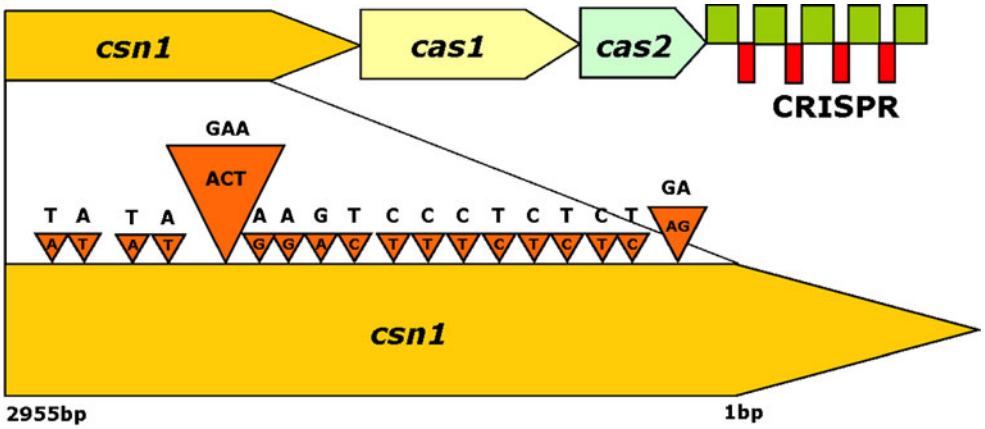
RFLP Type →	1	2	3	4	Δ
GBS +	23	3	0	0	0
GBS -	74	35	5	4	2
cst-II +	52	8	2	0	0
cst-II -	45	30	3	4	2

Fig. 5 *cas1* gene polymorphisms associate with *cst*-II gene presence and GBS. Bioinformatic analysis of *cas1* (Gene ID: 905808, 892 bp) using the *C. jejuni* reference sequences revealed DNA polymorphisms that enabled or disabled digestion with the restriction enzyme *Tsp*509I. In the *orange triangle*, the nucleotides are shown that were observed to be mutated in *cas1*. Above the orange triangles, the nucleotides are shown corresponding to the observed mutation that enabled or disabled *Tsp*509I digestion at the nucleotide recognition site AATT. For *cas1*, the mutations were found at positions 73, 197, 530, 556, 640, 751, 759, 775 and 793 bp from the gene start site. An overview is given of the four different PCR-RFLP types detected for the *cas1* gene as visualised on a 3 % agarose gel. *Numbers 1 to 4* correspond to the PCR-RFLP types detected for *cas1*. PCR-RFLP type Δ corresponds to a negative PCR reaction for *cas1*. PCR-RFLP types were analysed for an association with GBS only or for the present of the exchangeable *cst*-II gene. PCR-RFLP type 1 correlates with the presence of the *cst*-II gene (Fisher's exact probability test, $p=0.00016$), whereas PCR-RFLP type 2 correlates with the absence of the *cst*-II gene (Fisher's exact probability test, $p=0.0021$). PCR-RFLP type 1 associates with GBS-inducing *C. jejuni* isolates (Fisher's exact probability test, $p=0.011$) and correlated specifically with the *cst*-II-containing LOS class A gene locus (Fisher's exact probability test, $p<0.0001$)

and GD1 structures, since wild-type and *csn1* mutants both demonstrated an increase in OD_{600nm} overtime, displaying normal growth in Mueller Hinton broth. Next, the 11168Δ*csn1* mutant in comparison to the NCTC11168 wild-type isolate also demonstrated susceptibility to three (14 %) additional lytic *C. jejuni*-specific bacteriophages (*Cje*006, *Cje*009 and NCTC12675) in a bacteriophage plaque assay using the 29 different lytic *C. jejuni*-specific bacteriophages (Fig. 2). Noteworthy, for the NCTC12675 bacteriophage, it is previously demonstrated that the *C. jejuni* flagella are used as a receptor to establish an infection [47]. This same assay revealed that the inactivation of *csn1* in GB2, GB11 and GB19 had only a minor effect on bacteriophage susceptibility; establishing the importance of ganglioside-like structures GM1 and GD1 in bacteriophage defence (Fig. 2). Remarkably, the loss of *csn1* in GB2 and GB19 did result in strong susceptibility to the NCTC12681 bacteriophage and resistance to the *Cje*006 bacteriophage, respectively. Noteworthy, for the NCTC12681 bacteriophage, it is previously demonstrated that the *C. jejuni* flagella are used as a receptor to establish an infection [47].

C. jejuni cas2 and *cas1* gene polymorphisms associate with sialyltransferase Cst-II presence and GBS

The fact that some *C. jejuni* isolates lack the mobile LOS loci harbouring the *cst*-II gene made us explore whether specific CRISPR spacer sequences correlated with the absence of *cst*-II. The CRISPR region of 85 *C. jejuni* isolates was amplified, sequenced and analysed for complementarities with the mobile LOS loci harbouring *cst*-II. Although spacer sequences identical to the *cst*-II gene sequence in the CRISPR region of 85 *C. jejuni* isolates were not found (Supplemental Results 1), it was observed that four CRISPR spacers revealed 100 % identity with known *C. jejuni* (pro-)phage sequences (Fig. 3). The absence of *cst*-II-specific CRISPR spacers led us to investigate whether the *cas* genes harboured differences correlating to the presence of the *cst*-II gene. PCR-RFLP analysis of *cas2* and *cas1* genes in 26 GBS-associated and 120 matched enteritis-inducing isolates demonstrated that polymorphisms specific to each group were, indeed, present (*cas2*: $p=0.011$; *cas1*: $p=0.00016$). Remarkably, in *cas2*, a polymorphism was specifically associated with the LOS class E gene locus, which lacks *cst*-II ($p<0.0001$). Importantly, a specific polymorphism



DNA SNP associating with the *cst*-II gene [a]	Position from start [b]	Amino acid change [c]	*p* value [d]
GA	426bp	Gly → Ser	0.027
C	750bp	No change	0.027
T	804bp	No change	0.027
C	820bp	IsoLeu → Leu	0.027
T	1077bp	No change	0.0042
C	1080bp	No change	0.033
T	1125bp	No change	0.033
T	1152bp	No change	0.0042
T	1674bp	No change	0.001
C	1677bp	No change	0.001
A	1756bp	No change	0.003
G	1795bp	No change	0.0002
A	1821bp	Ala → Thr	0.003
ACT	1837 - 1839bp	Thr → Glu	0.003
T	2058bp	No change	0.011
A	2073bp	No change	0.0016
T	2716bp	Phe → Leu	0.0016
A	2726bp	Tyr → Phe	0.0016

Fig. 6 *csn1* polymorphisms associate with *cst*-II gene presence. A subset of our strain collection (9 GBS versus 18 enteritis isolates) supplemented with three GBS, HB9313, 260.94 and ICDCCJ7001 (i-CCJ7001), and five enteritis *C. jejuni* reference isolates, 11168, 81116, 8425, RM1221 and CG8486, obtained from the NCBI database were used for sequence analysis. The presence or absence of the *cst*-II gene was established for all used isolates by LOS class PCR: **a** shows the polymorphisms that associate with the presence of the *cst*-II gene; **b** demonstrates from which position a specific polymorphism was detected from the *csn1* gene start site; **c** amino acid changes that occurred due to nucleic acid polymorphisms; **d** shows the significance of the observed polymorphisms in relation to the presence of the *cst*-II gene. A Fisher's exact probability test and a contingency table were used to test whether the presence of the *cst*-II gene was associated with a specific polymorphism observed after sequencing

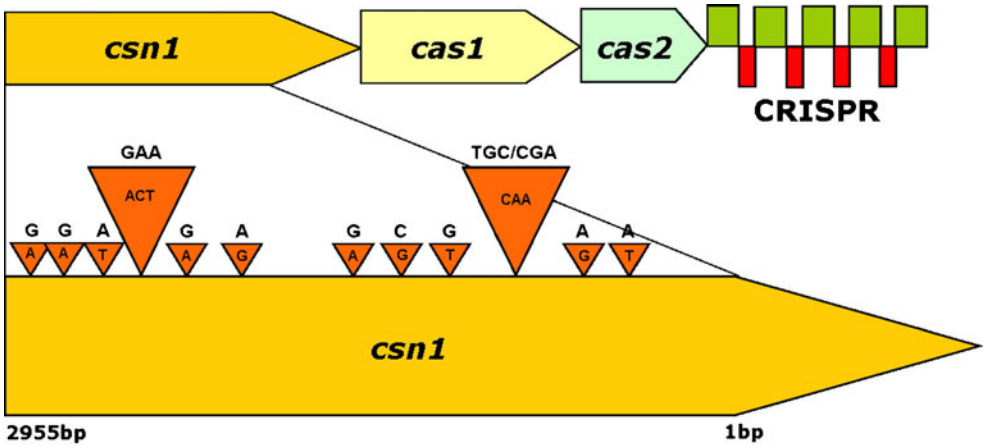
in *cas1* was detected more frequently in GBS-inducing isolates ($p=0.011$) and associated strongly with the presence of the LOS class A gene locus that harbours the *cst*-II gene and is associated with GBS [30] ($p<0.0001$) (Figs. 4 and 5).

Type II CRISPR-Cas marker gene *csn1* polymorphisms associate with *cst*-II presence and outcome of disease

The size of the *csn1* gene (2,955 bp) disabled a reliable use of our *Tsp*509I PCR-RFLP screening method for polymorphisms identification. Hence, we used PCR amplification and DNA sequencing to determine whether the *csn1* sequences from 12 GBS-associated and 23 enteritis-inducing clinical isolates of *C. jejuni* harboured polymorphisms that associated with the occurrence of the *cst*-II gene. Eighteen *csn1* (non)-synonymous polymorphisms were identified that correlated strongly with the presence of the *cst*-II gene (Fig. 6). Strikingly, 12 polymorphisms were over-represented in GBS-associating isolates, providing potential novel GBS markers. Five polymorphisms were further analysed by PCR and sequencing using a larger GBS and enteritis isolate collection, establishing that the observed polymorphisms, indeed, correlated with GBS ($p<0.05$) (Fig. 7).

Acquisition of *cst*-II is a more recent event than development of the CRISPR-Cas system

The observed effects of ganglioside-like structures GM1 and/or GD1 on CRISPR-Cas functionality in bacteriophage



DNA SNP associating with GBS [a]	Position from start [b]	Amino acid Change [c]	*p* value [d]	PCR or sequencing [e]
T	30bp	No change	0.037	
G	51bp	No change	0.037	
CAA	429 - 431bp	Ala → Lys or Glu	0.0006	0.00015 *
T	604bp	Asp → Tyr	0.0006	
G	750bp	Thr → Ser	0.0006	
A	885bp	No change	0.0097	
G	1795bp	No change	0.021	
A	1821bp	Ala → Thr	0.061	
ACT	1837 - 1839bp	Thr → Glu	0.069	0.023 *
T	2058bp	No change	0.010	0.029 **
A	2073bp	No change	0.014	0.037 **
A	2092bp	Thr → Ala	0.27	0.011 **

Fig. 7 *csn1* polymorphisms associate with GBS isolates: **a** shows the detected polymorphisms that associate specifically with GBS-inducing isolates; **b** reveals from which position a specific polymorphism was detected, counting nucleotides from the *csn1* gene start site; **c** amino acid changes that occurred due to a polymorphism; **d** shows the significance after analysing 35 *csn1* sequences on GBS-associating polymorphisms using a contingency table and a Fisher's exact probability test; **e** *For a subset of the sequenced-detected polymorphisms, a PCR analysis was performed on our Dutch and complete GBS and enteritis strain collection (Dutch collection consists of 26 GBS- and 120 matched enteritis-inducing isolates; the complete collection consists of 60 four GBS- and 175 enteritis-inducing isolates) to confirm the association with GBS; CAA PCR-positive in Dutch GBS isolates 5:21 and matched enteritis isolates 5:115 (Fisher's exact probability test, $p=0.016$); CAA PCR-positive in the complete GBS collection 17:47 and in the enteritis collection 14:161 (Chi-square test; $p=0.00015$); GAA PCR-positive in GBS 11:53 in enteritis 56:119 (Chi-square test; $p=0.023$). **Sequencing of 30 Dutch GBS-inducing and 69 matched enteritis-inducing isolates confirmed the single DNA polymorphisms T (2,058 bp) in GBS-positive 24:2 in enteritis 49:20 (Chi-square test; $p=0.029$), A (2,073 bp) in GBS-positive 24:2 in enteritis 50:19 (Chi-square test; $p=0.037$) and A (2,092 bp) in GBS-positive 20:6 in enteritis 33:36 (Chi-square test; $p=0.011$)

defence, the presence of *cas* gene mutations, even frameshifts in the *cas* genes of the isolates 260.94 (taxid: 360108), CG8486 (taxid: 398000) and ICDCCJ07001 (taxid: 757425) as presented in the NCBI database and a degenerated CRISPR element in the presence of sialylation all potentially suggests an evolutionary link. We hypothesised that *C. jejuni* isolates with GM1-and/or GD1-mediated bacteriophage resistance are no longer under selection pressure to maintain a functional CRISPR-Cas system for bacteriophage defence. This scenario implies that *cas* gene polymorphisms are indicative for a group of *C. jejuni* isolates that might have emerged more recently. Molecular clock bioinformatic analysis using CRISPR and the *cas* gene sequence indeed confirmed that *cst*-II-positive *C. jejuni* isolates display a shorter time interval to the most recent common ancestor and, thus, appeared to have originated more recently compared to isolates that lack *cst*-II (Table 2).

Table 2 Acquisition of *cst*-II is a more recent event than development of the CRISPR-Cas system

Statistic	CRISPR		*csn1* (*Cj*1523)	
	Mean	ESS	Mean	ESS
posterior	−1.067E4	2255.232	−5,908.089	7,064.556
prior	-240.972	1,675.431	97.007	6424.113
treeModel.rootHeight	33.263	5,771.718	1.703E−2	6,914.826
Tmrca (*cst*-II −)	33.263	5,771.718	1.703E−2	6,916.024
Tmrca (*cst*-II +)	6.211	1,720.612	1.56E−2	7,974.467
constant.popSize	7.613	3,574.551	2.783E−2	1.464E4
kappa	1.52	5,817.852	8.827	4,608.072
clock.rate	1	–	1	–
treeLikelihood	−1.043E4	2,508.353	−6,005.096	5,302.258
coalescent	−235.368	1,675.729	98.84	6,715.408

The BEAST software models relevant population genetic parameters using Bayesian statistics. The lower limit for sufficient statistical reliability, 200, indicated by the effective sample size (ESS) value, is exceeded by at least 5–6 times for all estimated parameters. The statistics that reflect the reliability of the BEAST models are the prior and posterior (which describe the appropriateness of the chosen model parameters, including the speciation model, and the reliability of the outcome of the population parameter statistics), the (model) likelihood and resulting phylogenetic tree likelihood. The treeModel.rootHeight parameter describes the evolutionary distance of taxa, represented by the time to the most recent common ancestor (TMRCA) to the root of a tree; taxa with larger mean rootheight values are older. The kappa value describes the transition/transversion ratio for each locus; the different values for CRISPR and *csn1* sequences suggests a different evolutionary age. The larger value for CRISPR rootheights and TMRCA indicates the older evolutionary age of CRISPR sequences compared to *csn1* sequences. The larger TMRCA for the *cst*-II-negative isolates for the CRISPR element indicates that the *cst*-II positive isolates have originated later than the *cst*-II-negative isolates

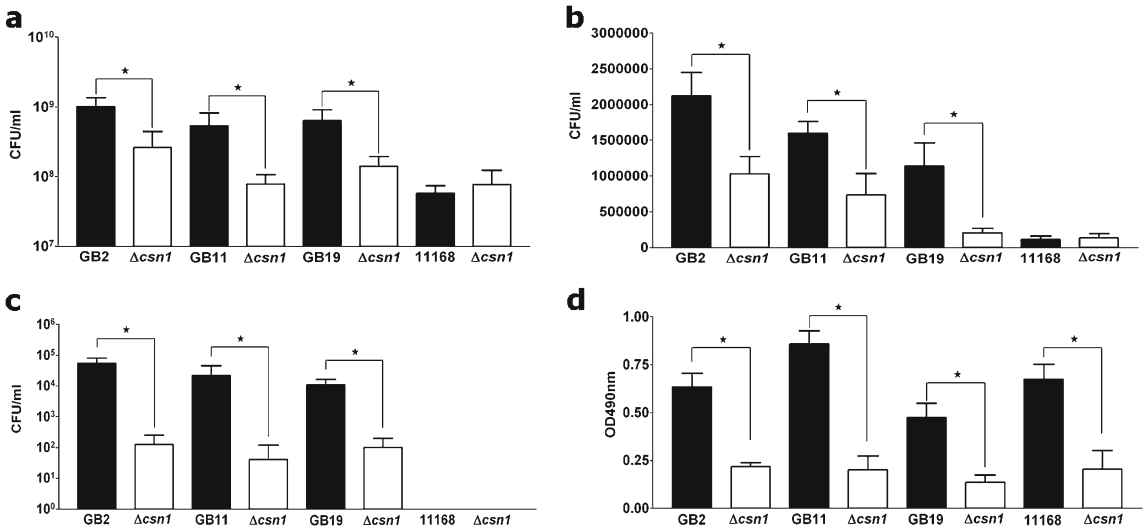


Fig. 8 *csn1* is involved in the pathogenic features of mainly the GBS-inducing isolates. *C. jejuni* wild-type strains GB2, GB11, GB19, NCTC11168 and their Δ*csn1* mutant variants were studied on: **a** adhesion onto; **b** invasion into; and **c** translocation across human Caco-2 cells. Colony forming units (CFU) were calculated per ml. Data are expressed as the standard error of the mean (SEM) of three independent experiments, each performed in duplicate; **d** Cytotoxic effects of GB2, GB11, GB19, NCTC11168 and their Δ*csn1* mutant variants on HT-29 cells were measured at OD_{490nm} visualising the LDH release in cell culture supernatants. The data are expressed as the SEM of triplicate determinations performed in triplicate. Significant differences between GBS wild-type and Δ*csn1* mutants were observed. In the adhesion and invasion assay, this significance was $*p<0.05$ and in the translocation and cytotoxicity assays $*p<0.01$ using a paired *t*-test between wild-type and Δ*csn1* mutants, respectively. Notable, the NCTC11168 isolate and its *csn1* mutant were not able to translocate

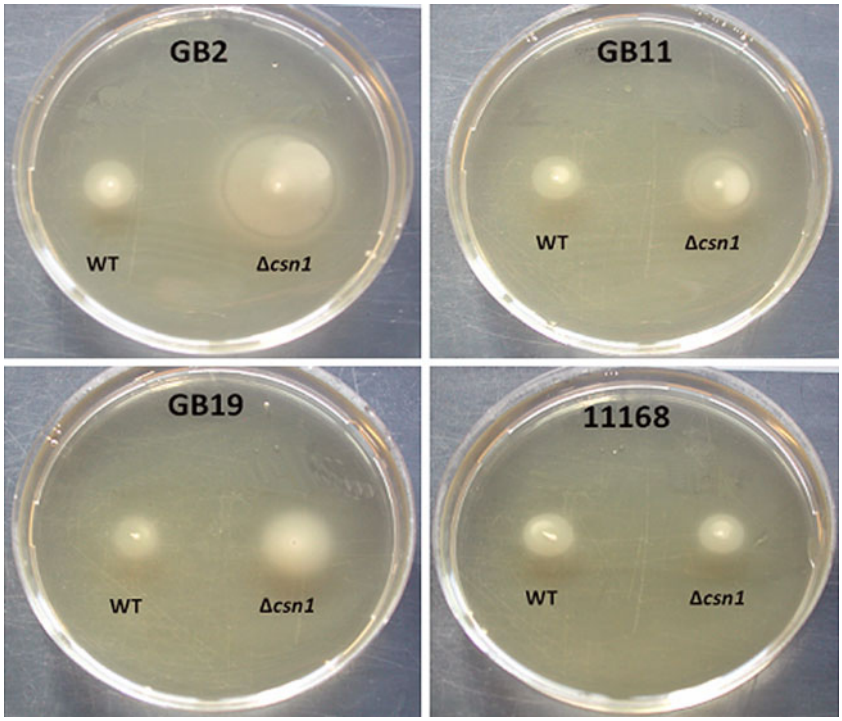


Fig. 9 *csn1* knock-out mutagenesis influences swarming behaviour of the GBS-inducing isolates. The figure shows a representative swarming picture observed in three independent experiments with four wild-type strains GB2, GB11, GB19, NCTC11168 and their isogenic Δ*csn1* mutants. Swarming circle diameters were measured in millimetres±the standard error of the mean (SEM)

Deletion of *csn1* affects *C. jejuni* virulence and GBS serum antibody binding to ganglioside-like structures

The majority of bacteria belonging to the type II CRISPR-Cas subgroup are bacterial pathogens [33]. Hence, we, therefore, assessed whether the type II CRISPR-Cas marker gene *csn1* played a role in virulence in three *cst*-II-positive GBS isolates, GB2, GB11 and GB19, and one *cst*-II-negative isolate, NCTC11168. Comparison of our GB2, GB11, GB19 and NCTC11168 wild-type isolates against their isogenic Δ*csn1* mutants in infection assays using Caco-2 cells showed only for the GBSΔ*csn1* mutants a significant decrease in adherence to, invasion in and translocation across Caco-2 cell monolayers (Fig. 8a–c). The inactivation of *csn1* in GB2, GB11, GB19 and NCTC11168 abrogated epithelial cell damage, as measured by cellular lactate dehydrogenase (LDH) release, a marker of host cell necrosis (Fig. 8d). Next, we observed that the swarming phenotype for GB2, GB11 and GB19 increased through loss of Csn1 (Fig. 9). We measured for GB2 12±0.3; GB2Δ*csn1* 30±0.6; GB11 11.5±0.2; GB11Δ*csn1* 20±0.6; GB19 11±0.5; GB19Δ*csn1* 22±0.4; NCTC11168 9±0.7 and NCTC11168Δ*csn1* 9±0.3. All three GBSΔ*csn1* mutants showed an increased swarming circle compared to their respective wild-type parent isolates (Fig. 9). No differences in swarming were observed between the NCTC11168 wild-type isolate and its isogenic Δ*csn1* mutant (Fig. 9). Supplementation of *csn1* from the GBS isolate GB11 into a *C. jejuni* isolate lacking CRISPR-Cas but harbouring *cst*-II and ganglioside-like LOS established that *csn1* affects *C. jejuni* virulence, as observed in the GBS isolates by increasing the invasion capacity but reducing swarming (Fig. 10).

GBS patients develop antibodies that cross-react with both bacterial cell envelope-bound ganglioside-like structures and gangliosides on the human peripheral nerve, leading to nerve destruction [28]. The link between type II CRISPR-Cas marker gene *csn1*, sialyltransferase presence, ganglioside-like structure expression and GBS made us address whether the presence or absence of *csn1* affects the serum antibody binding to the *C. jejuni* sialylated cell envelope. A Western blot analysis using GBS patient serum revealed a remarkable increase in the binding of IgG serum antibodies to the cell envelope-bound ganglioside-like structures of the three GBSΔ*csn1* mutants (Fig. 11). The NCTC11168 isolate and its Δ*csn1* mutant did not reveal any binding of IgG serum antibodies to its LOS structures (Fig. 11).

Discussion

In this study, we highlighted several novel aspects of the type II CRISPR-Cas system in ganglioside-like LOS expressing pathogenic bacteria. We revealed in *C. jejuni* that cell envelope-bound ganglioside-like structures GM1 and GD1 are an efficient bacteriophage defence system. For type II CRISPR-Cas we demonstrated that this system plays only a minor role in bacteriophage defence in Cst-II-positive *C. jejuni* isolates. In contrast, this system is more active in providing bacteriophage resistance in the NCTC11168 *C. jejuni* isolate, which lacks the Cst-II-expressed GM1 and

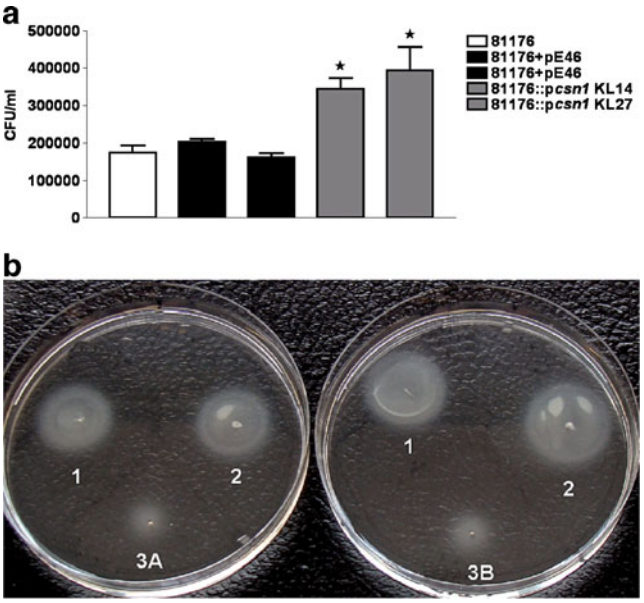


Fig. 10 *csn1* supplementation confirms the involvement of Csn1 in *C. jejuni* virulence: **a** *C. jejuni* wild-type strain 81176; a control strain (81176E) harbouring an empty vector pE46 used for *csn1* supplementation and two 81176 clones supplemented with the pE46 vector containing the *csn1* gene and *csn1* promoter from the GB11 GBS isolate (KL14 and Kl27) were studied for invasion into the Caco-2 cells using a gentamicin exclusion assay. Data are expressed as the SEM of at least three independent experiments, each performed in duplicate (paired *t*-test, $*p<0.05$); **b** *C. jejuni* wild-type strain 81176 (1); a control strain (81176E) harbouring an empty vector pE46 used for *csn1* supplementation (2); and the two 81176 clones supplemented with the pE46 vector containing the *csn1* gene and *csn1* promoter from the GB11 isolate were studied on swarming behaviour; (3A) is Kl14 and (3B) is KL27. The assay was repeated three times and the average diameter±SEM was: for 81176, 28±3 mm; 81176E, 27±2 mm; Kl14, 12±4 mm; and Kl27, 10±2 mm

GD1 ganglioside-like structures. Next, we observed that this coincided mainly in the Cst-II-positive *C. jejuni* isolates with a functionality change of the type II CRISPR-Cas marker gene *csn1* towards a role(s) in virulence. Finally, new *C. jejuni* GBS markers were discovered in the *cas* genes, which is associated with the presence of the earlier established GBS marker *cst*-II [29] or were novel and *cas* gene-specific.

In 2010, Bruce Levin presented a mathematical model on bacterial CRISPR-Cas dynamics, which predicted that cell envelope-mediated bacteriophage resistance could affect the preservation of CRISPR-Cas [46]. Using *C. jejuni* as a model, we were able to provide evidence for a very basic prediction made in the mathematical model of Levin. We demonstrated with different techniques that the acquisition of an alternative bacteriophage defence system in *jejuni*, in our case ganglioside-like LOS, correlates with a degenerated CRISPR-Cas system.

While ganglioside-like structures provide bacteriophage resistance in *C. jejuni*, next to CRISPR-Cas, it is certainly not the only mechanism [57, 58]. For example, in this study, we also demonstrated that isolates harbouring an LOS class E locus were effectively protected against the tested bacteriophages that use the cell envelope as a receptor [47]. In contrast to these results, we were able to show that class E-expressed LOS on *C. jejuni* is not as efficient in defence as class A-expressed ganglioside-like LOS, in view of the observed higher levels of NCTC bacteriophage resistance in isolates with GM1 and GD1 expression.

As both ganglioside-like LOS expression and type II CRISPR-Cas occur in other bacterial pathogens, e.g. *N. meningitidis*, *Haemophilus* spp. and *P. multocida*, we used comparative genomics to investigate whether the link between the presence of sialyltransferases and a reduced type II CRISPR-Cas was not specific to *C. jejuni* only. Although not yet backed up by experimental data in *N. meningitidis*, *Haemophilus* spp. and *P. multocida*, we did see a similar pattern as in *C. jejuni*, with the presence of a sialyltransferase gene being linked to the degeneration of CRISPR-Cas (Fig. 1).

In the present study, we observed that a reduced or absent CRISPR array and (non)-synonymous mutations in the *cas* genes were associated with the presence of the chromosomally integrated mobile *cst*-II containing loci and ganglioside-

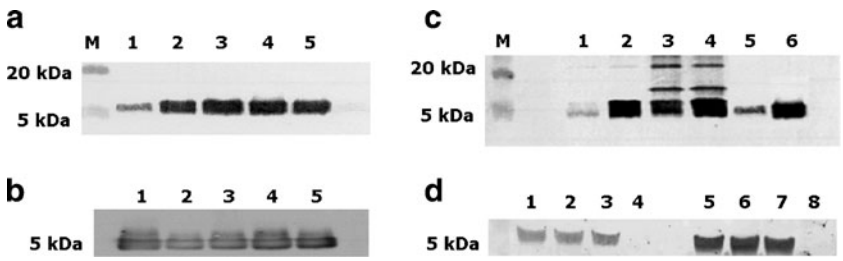


Fig. 11 Increased serum antibody recognition of ganglioside-like LOS of the GBS*Δcsn1* mutants; **a** GBS patient serum antibody binding between GB11 wild-type (1) and four GB11*Δcsn1* mutant clones (2, 3, 4 and 5) was compared. Samples reveal an increase of GBS patient serum antibody binding against ganglioside-like LOS of the GB11*Δcsn1* variants (2, 3, 4 and 5); **b** cholera toxin confirms equal loading for (**a**); **c** presence for ganglioside-like LOS was analysed in the supernatant of culture broth medium, (1) GB11 wild-type and (2) GB11*Δcsn1* mutant, and from the lysates generated from the (3) GB11 wild-type and (4) GB11*Δcsn1* mutant; proteinase K-treated lysates (5) GB11 wild-type and (6) GB11*Δcsn1* mutant; **d** GBS patient serum antibody binding of the GBS wild-type isolates (1) GB2, (2) GB11, (3) GB19 and (4) the NCTC11168 enteritis isolate were compared to their *Δcsn1* mutants (5) GB2*Δcsn1*, (6) GB11*Δcsn1*, (7) GB19*Δcsn1* and (8) NCTC11168*Δcsn1*. A part of a pre-stained SDS-PAGE broad range standard (M) is shown in the range of 20 kDa and 5 kDa

like LOS expression. Earlier, the occurrence of *cst*-II and ganglioside-like LOS in *C. jejuni* was linked to increased intestinal epithelial invasion, severe gastro-enteritis, bloody diarrhea, reactive arthritis and GBS [9–11, 32, 59]. Hence, a reduced or absent CRISPR array, (non)-synonymous mutations in the *cas* genes that associate strongly with *cst*-II presence and ganglioside-like LOS expression could, thus, serve as useful molecular markers for *C. jejuni* isolates that are able to induce a more severe disease phenotype in humans. Interestingly, ganglioside-like structures and sialyltransferase presence in *N. meningitidis* and *H. influenzae* are also linked to increased virulence [5]. To be able to correlate the degeneration of CRISPR-Cas, ganglioside-like LOS expression and increased virulence in these bacterial species, further studies are required. Overall, our observations do not stand alone, since CRISPR-Cas degeneration was found in other studies to be of predictive value as to whether a bacterial strain would display increased virulence or even multi-drug resistance [60–64].

Earlier, for *P. aeruginosa*, it was shown that a CRISPR-Cas system, by itself, could serve a function in virulence, since biofilm formation and motility changes were triggered in this bacterium by interactions between the CRISPR element and a chromosomally integrated mobile element [65, 66]. On the contrary to the *Pseudomonas* study, we did not find any indications in this study that interactions between the chromosomally integrated mobile *cst*-II-containing loci and the *C. jejuni* CRISPR element occurred. In the GM1- and/or GD1-expressing isolates, the chances of such an interaction are also limited due to the low number or absence of CRISPR spacers, but we cannot exclude from this study that such interactions do take place.

The mammalian-associated pathogens, such as *C. jejuni*, all harbour the type II CRISPR-Cas marker gene *csn1* [33, 34]. Recently, in *Streptococcus pyogenes* Csn1 was shown to be involved together with RNase III in one of the steps required for successful bacteriophage defence, namely, processing of the CRISPR precursor RNA [67]. In *C. jejuni*, we also observed the involvement of Csn1 in bacteriophage defence, but our results go even further by showing, for the first time, that this protein is involved in virulence. Next to that, loss of Csn1 in three GBS-inducing Cst-II-positive *C. jejuni* isolates not only lead to a decrease in virulence, but also to an increase in the binding of IgG-specific antibodies to ganglioside-like structures GM1 and GD1, which are primarily detected in GBS patient serum [30, 68, 69].

The results on antibody binding could be indicative that Csn1 plays a role in mediating cell envelope topology or composition in *C. jejuni*. Earlier, in *E. coli*, a relationship between CRISPR-Cas and the cell envelope has been established [70] by showing that bacterial immunity is linked to bacterial membrane stresses [71]. Remarkably, for the *C. jejuni* isolate NCTC11168, it was earlier established that genes involved in the expression of cell envelope structures, mainly LOS and capsule, together with *csn1* (*Cj*1523), were differentially expressed after passage through a mouse intestine [72]. Further studies are required to reveal the mechanism(s) of action explaining the role of *csn1* in *C. jejuni* virulence. Whether this gene plays a role in virulence next to bacteriophage defence in the other type II CRISPR-Cas mammalian-associated pathogens remains to be elucidated.

Based on our results, we hypothesise that, in *C. jejuni*, ganglioside-like LOS is evolutionarily replacing CRISPR-Cas in bacteriophage defence, resulting in the observed effects on CRISPR-Cas in this bacterium. We conclude that there is a distinct link between *C. jejuni* bacteriophage defence, virulence and GBS. Overall, our report suggests that the type II CRISPR-Cas system in *C. jejuni* harbours potential clinically relevant features.

Acknowledgements This work was supported by a grant from the Human Frontier Science Program (RGP 38/2003) and by the BBSRC Institute Strategic Programme Grant to the IFR. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. We would like to thank Theo Hoogenboezem (Erasmus MC) for the mass spectrometry analysis of the recombinant Csn1 protein for polyclonal antibody production and Ian Connerton for bacteriophage CP28.

Conflicts of interest Alex van Belkum is an employee of bioMérieux. There are no conflicts of interest to disclose.

References

1. Guerrant RL, Hughes JM, Lima NL, Crane J (1990) Diarrhea in developed and developing countries: magnitude, special settings, and etiologies. Rev Infect Dis 12(Suppl 1):S41–S50
2. Stephens DS (2009) Biology and pathogenesis of the evolutionarily successful, obligate human bacterium *Neisseria meningitidis*. Vaccine 27(Suppl 2):B71–B77
3. Harrison LH, Simonsen V, Waldman EA (2008) Emergence and disappearance of a virulent clone of *Haemophilus influenzae* biogroup aegyptius, cause of Brazilian purpuric fever. Clin Microbiol Rev 21(4):594–605
4. Nakwan N, Nakwan N, Atta T, Chokephaibulkit K (2009) Neonatal pasteurellosis: a review of reported cases. Arch Dis Child Fetal Neonatal Ed 94(5):F373–F376
5. Severi E, Hood DW, Thomas GH (2007) Sialic acid utilization by bacterial pathogens. Microbiology 153(Pt 9):2817–2822
6. Guerry P, Ewing CP, Hickey TE, Prendergast MM, Moran AP (2000) Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. Infect Immun 68(12):6656–6662
7. Fox KL, Cox AD, Gilbert M, Wakarchuk WW, Li J, Makepeace K, Richards JC, Moxon ER, Hood DW (2006) Identification of a bifunctional lipopolysaccharide sialyltransferase in *Haemophilus influenzae*: incorporation of disialic acid. J Biol Chem 281 (52):40024–40032
8. Tatum FM, Tabatabai LB, Briggs RE (2009) Sialic acid uptake is necessary for virulence of *Pasteurella multocida* in turkeys. Microb Pathog 46(6):337–344

9. Habib I, Louwen R, Uyttendaele M, Houf K, Vandenberg O, Nieuwenhuis EE, Miller WG, van Belkum A, De Zutter L (2009) Correlation between genotypic diversity, lipooligosaccharide gene locus class variation, and caco-2 cell invasion potential of *Campylobacter jejuni* isolates from chicken meat and humans: contribution to virulotyping. Appl Environ Microbiol 75(13):4277–4288
10. Louwen R, Heikema A, van Belkum A, Ott A, Gilbert M, Ang W, Endtz HP, Bergman MP, Nieuwenhuis EE (2008) The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion. Infect Immun 76 (10):4431–4438
11. Perera VN, Nachamkin I, Ung H, Patterson JH, McConville MJ, Coloe PJ, Fry BN (2007) Molecular mimicry in *Campylobacter jejuni*: role of the lipo-oligosaccharide core oligosaccharide in inducing anti-ganglioside antibodies. FEMS Immunol Med Microbiol 50(1):27–36
12. Unkmeir A, Kämmerer U, Stade A, Hübner C, Haller S, Kolb-Mäurer A, Frosch M, Dietrich G (2002) Lipooligosaccharide and polysaccharide capsule: virulence factors of *Neisseria meningitidis* that determine meningococcal interaction with human dendritic cells. Infect Immun 70(5):2454–2462
13. Hammerschmidt S, Hilse R, van Putten JP, Gerardy-Schahn R, Unkmeir A, Frosch M (1996) Modulation of cell surface sialic acid expression in *Neisseria meningitidis* via a transposable genetic element. EMBO J 15(1):192–198
14. Chiu CP, Watts AG, Lairson LL, Gilbert M, Lim D, Wakarchuk WW, Withers SG, Strynadka NC (2004) Structural analysis of the sialyltransferase CstII from *Campylobacter jejuni* in complex with a substrate analog. Nat Struct Mol Biol 11(2):163–170
15. Gilbert M, Karwaski MF, Bernatchez S, Young NM, Taboada E, Michniewicz J, Cunningham AM, Wakarchuk WW (2002) The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. J Biol Chem 277(1):327–337
16. Thon V, Lau K, Yu H, Tran BK, Chen X (2011) PmST2: a novel *Pasteurella multocida* glycolipid alpha2-3-sialyltransferase. Glycobiology 21(9):1206–1216
17. Packiam M, Shell DM, Liu SV, Liu YB, McGee DJ, Srivastava R, Seal S, Rest RF (2006) Differential expression and transcriptional analysis of the alpha-2,3-sialyltransferase gene in pathogenic *Neisseria* spp. Infect Immun 74(5):2637–2650
18. Sadler F, Fox A, Neal K, Dawson M, Cartwright K, Borrow R (2003) Genetic analysis of capsular status of meningococcal carrier isolates. Epidemiol Infect 130(1):59–70
19. Tsai CM (2001) Molecular mimicry of host structures by lipooligosaccharides of *Neisseria meningitidis*: characterization of sialylated and nonsialylated lacto-N-neotetraose (Galbeta1-4GlcNAcbeta1-3Galbeta1-4Glc) structures in lipooligosaccharides using monoclonal antibodies and specific lectins. Adv Exp Med Biol 491:525–542
20. Harvey HA, Swords WE, Apicella MA (2001) The mimicry of human glycolipids and glycosphingolipids by the lipooligosaccharides of pathogenic *Neisseria* and *Haemophilus*. J Autoimmun 16(3):257–262
21. Ang CW, Jacobs BC, Laman JD (2004) The Guillain–Barré syndrome: a true case of molecular mimicry. Trends Immunol 25 (2):61–66
22. Brigham KS, Sandora TJ (2009) *Neisseria meningitidis*: epidemiology, treatment and prevention in adolescents. Curr Opin Pediatr 21(4):437–443
23. Yuki N (2007) Ganglioside mimicry and peripheral nerve disease. Muscle Nerve 35(6):691–711
24. Bennetto LP, Lyons P (2004) Miller Fisher syndrome associated with *Pasteurella multocida* infection. J Neurol Neurosurg Psychiatry 75(12):1786–1787
25. Mori M, Kuwabara S, Miyake M, Noda M, Kuroki H, Kanno H, Ogawara K, Hattori T (2000) *Haemophilus influenzae* infection and Guillain–Barré syndrome. Brain 123(Pt 10):2171–2178
26. van Doorn PA, Ruts L, Jacobs BC (2008) Clinical features, pathogenesis, and treatment of Guillain–Barré syndrome. Lancet Neurol 7(10):939–950
27. Willison HJ, Kennedy PG (1993) Gangliosides and bacterial toxins in Guillain–Barré syndrome. J Neuroimmunol 46(1–2):105–112
28. Kiefer R, Kieseier BC, Stoll G, Hartung HP (2001) The role of macrophages in immune-mediated damage to the peripheral nervous system. Prog Neurobiol 64(2):109–127
29. van Belkum A, van den Braak N, Godschalk P, Ang W, Jacobs B, Gilbert M, Wakarchuk W, Verbrugh H, Endtz H (2001) A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. Nat Med 7(7):752–753
30. Godschalk PC, Heikema AP, Gilbert M, Komagamine T, Ang CW, Glerum J, Brochu D, Li J, Yuki N, Jacobs BC, van Belkum A, Endtz HP (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain–Barré syndrome. J Clin Invest 114(11):1659–1665
31. Gilbert M, Godschalk PC, Karwaski MF, Ang CW, van Belkum A, Li J, Wakarchuk WW, Endtz HP (2004) Evidence for acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a patient with Guillain–Barré syndrome, by horizontal exchange. Infect Immun 72(2):1162–1165
32. Phongsisay V, Perera VN, Fry BN (2006) Exchange of lipooligosaccharide synthesis genes creates potential Guillain–Barré syndrome-inducible strains of *Campylobacter jejuni*. Infect Immun 74(2):1368–1372
33. Haft DH, Selengut J, Mongodin EF, Nelson KE (2005) A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Comput Biol 1(6):e60
34. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF, van der Oost J, Koonin EV (2011) Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9(6):467–477
35. Deveau H, Garneau JE, Moineau S (2010) CRISPR/Cas system and its role in phage–bacteria interactions. Annu Rev Microbiol 64:475–493
36. Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. Science (New York, N.Y.) 327(5962):167–170
37. Stevens J, Chen LM, Carney PJ, Garten R, Foust A, Le J, Pokorny BA, Manojkumar R, Silverman J, Devis R, Rhea K, Xu X, Bucher DJ, Paulson JC, Cox NJ, Klimov A, Donis RO (2010) Receptor specificity of influenza A H3N2 viruses isolated in mammalian cells and embryonated chicken eggs. J Virol 84(16):8287–8299
38. Herrler G, Klenk HD (1987) The surface receptor is a major determinant of the cell tropism of influenza C virus. Virology 159(1):102–108
39. Superti F, Girmenta C, Seganti L, Orsi N (1986) Role of sialic acid in cell receptors for vesicular stomatitis virus. Acta Virol 30(1):10–18
40. Superti F, Hauttecoeur B, Morelec MJ, Goldoni P, Bizzini B, Tsiang H (1986) Involvement of gangliosides in rabies virus infection. J Gen Virol 67(Pt 1):47–56
41. Gilbert J, Dahl J, Riney C, You J, Cui C, Holmes R, Lencer W, Benjamin T (2005) Ganglioside GD1a restores infectibility to mouse cells lacking functional receptors for polyomavirus. J Virol 79(1):615–618
42. Superti F, Donelli G (1991) Gangliosides as binding sites in SA-11 rotavirus infection of LLC-MK2 cells. J Gen Virol 72(Pt 10):2467–2474
43. Markwell MA, Fredman P, Svennerholm L (1984) Receptor ganglioside content of three hosts for Sendai virus. MDBK, HeLa, and MDCK cells. Biochim Biophys Acta 775(1):7–16

44. Sinibaldi L, Goldoni P, Pietropaolo V, Longhi C, Orsi N (1990) Involvement of gangliosides in the interaction between BK virus and Vero cells. Arch Virol 113(3–4):291–296
45. Yao L, Korteweg C, Hsueh W, Gu J (2008) Avian influenza receptor expression in H5N1-infected and noninfected human tissues. FASEB J 22(3):733–740
46. Levin BR (2010) Nasty viruses, costly plasmids, population dynamics, and the conditions for establishing and maintaining CRISPR-mediated adaptive immunity in bacteria. PLoS Genet 6(10):e1001171
47. Coward C, Grant AJ, Swift C, Philp J, Towler R, Heydarian M, Frost JA, Maskell DJ (2006) Phase-variable surface structures are required for infection of *Campylobacter jejuni* by bacteriophages. Appl Environ Microbiol 72(7):4638–4647
48. Connerton PL, Loc Carrillo CM, Swift C, Dillon E, Scott A, Rees CE, Dodd CE, Frost J, Connerton IF (2004) Longitudinal study of *Campylobacter jejuni* bacteriophages and their hosts from broiler chickens. Appl Environ Microbiol 70(7):3877–3883
49. Godschalk PC, Kuijf ML, Li J, St Michael F, Ang CW, Jacobs BC, Karwaski MF, Brochu D, Moterassed A, Endtz HP, van Belkum A, Gilbert M (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain–Barré and Miller Fisher syndromes. Infect Immun 75(3):1245–1254
50. Dzieciatkowska M, Liu X, Heikema AP, Houliston RS, van Belkum A, Schweda EK, Gilbert M, Richards JC, Li J (2008) Rapid method for sensitive screening of oligosaccharide epitopes in the lipooligosaccharide from *Campylobacter jejuni* strains isolated from Guillain–Barré syndrome and Miller Fisher syndrome patients. J Clin Microbiol 46(10):3429–3436
51. Grissa I, Vergnaud G, Pourcel C (2007) CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic Acids Res 35(Web Server issue):W52–W57
52. Wren BW, Henderson J, Ketley JM (1994) A PCR-based strategy for the rapid construction of defined bacterial deletion mutants. Biotechniques 16(6):994–996
53. van Vliet AH, Wooldridge KG, Ketley JM (1998) Iron-responsive gene regulation in a *Campylobacter jejuni* fur mutant. J Bacteriol 180(20):5291–5298
54. Kim JS, Li J, Barnes IH, Baltzegar DA, Pajaniappan M, Cullen TW, Trent MS, Burns CM, Thompson SA (2008) Role of the *Campylobacter jejuni Cj*1461 DNA methyltransferase in regulating virulence characteristics. J Bacteriol 190(19):6524–6529
55. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214
56. MacCallum A, Hardy SP, Everest PH (2005) *Campylobacter jejuni* inhibits the absorptive transport functions of Caco-2 cells and disrupts cellular tight junctions. Microbiology 151(7):2451–2458
57. Gaasbeek EJ, Wagenaar JA, Guilhabert MR, van Putten JP, Parker CT, van der Wal FJ (2010) Nucleases encoded by the integrated elements CJIE2 and CJIE4 inhibit natural transformation of *Campylobacter jejuni*. J Bacteriol 192(4):936–941
58. Gaasbeek EJ, Wagenaar JA, Guilhabert MR, Wösten MM, van Putten JP, van der Graaf-van Bloois L, Parker CT, van der Wal FJ (2009) A DNase encoded by integrated element CJIE1 inhibits natural transformation of *Campylobacter jejuni*. J Bacteriol 191 (7):2296–2306
59. Mortensen NP, Kuijf ML, Ang CW, Schiellerup P, Krogfelt KA, Jacobs BC, van Belkum A, Endtz HP, Bergman MP (2009) Sialylation of *Campylobacter jejuni* lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. Microbes Infect 11(12):988–994
60. Lindenstrauss AG, Pavlovic M, Bringmann A, Behr J, Ehrmann MA, Vogel RF (2011) Comparison of genotypic and phenotypic cluster analyses of virulence determinants and possible role of CRISPR elements towards their incidence in *Enterococcus faecalis* and *Enterococcus faecium*. Syst Appl Microbiol 34(8):553–560
61. Delaney NF, Balenger S, Bonneaud C, Marx CJ, Hill GE, Ferguson-Noel N, Tsai P, Rodrigo A, Edwards SV (2012) Ultrafast evolution and loss of CRISPRs following a host shift in a novel wildlife pathogen, *Mycoplasma gallisepticum*. PLoS Genet 8(2): e1002511
62. Palmer KL, Gilmore MS (2010) Multidrug-resistant enterococci lack CRISPR-cas. MBio 1(4). Pii: e00227-10
63. Nozawa T, Furukawa N, Aikawa C, Watanabe T, Haobam B, Kurokawa K, Maruyama F, Nakagawa I (2011) CRISPR inhibition of prophage acquisition in *Streptococcus pyogenes*. PLoS One 6 (5):e19543
64. Fricke WF, Mammel MK, McDermott PF, Tartera C, White DG, Leclerc JE, Ravel J, Cebula TA (2011) Comparative genomics of 28 *Salmonella enterica* isolates: evidence for CRISPR-mediated adaptive sublineage evolution. J Bacteriol 193(14):3556–3568
65. Palmer KL, Whiteley M (2011) DMS3-42: the secret to CRISPR-dependent biofilm inhibition in *Pseudomonas aeruginosa*. J Bacteriol 193(14):3431–2
66. Zegans ME, Wagner JC, Cady KC, Murphy DM, Hammond JH, O'Toole GA (2009) Interaction between bacteriophage DMS3 and host CRISPR region inhibits group behaviors of *Pseudomonas aeruginosa*. J Bacteriol 191(1):210–219
67. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471(7340):602–607
68. Ho TW, Willison HJ, Nachamkin I, Li CY, Veitch J, Ung H, Wang GR, Liu RC, Cornblath DR, Asbury AK, Griffin JW, McKhann GM (1999) Anti-GD1a antibody is associated with axonal but not demyelinating forms of Guillain–Barré syndrome. Ann Neurol 45 (2):168–173
69. Sinha S, Prasad KN, Jain D, Pandey CM, Jha S, Pradhan S (2007) Preceding infections and anti-ganglioside antibodies in patients with Guillain–Barré syndrome: a single centre prospective case–control study. Clin Microbiol Infect 13(3):334–337
70. Perez-Rodriguez R, Haitjema C, Huang Q, Nam KH, Bernardis S, Ke A, DeLisa MP (2011) Envelope stress is a trigger of CRISPR RNA-mediated DNA silencing in *Escherichia coli*. Mol Microbiol 79(3):584–599
71. Raivio T (2011) Identifying your enemies—could envelope stress trigger microbial immunity? Mol Microbiol 79(3):557–561
72. Jerome JP, Bell JA, Plovanich-Jones AE, Barrick JE, Brown CT, Mansfield LS (2011) Standing genetic variation in contingency loci drives the rapid adaptation of *Campylobacter jejuni* to a novel host. PLoS One 6(1):e16399