Novel *Campylobacter concisus* Lipooligosaccharide is a Determinant of Inflammatory Potential and Virulence

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Running title*: Campylobacter concisus*: Lipooligosaccharide and inflammation

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Abbreviations: GI, gastrointestinal; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; GlcN3N, 2,3-diamino-2,3-dideoxy-D-glucose; Hex, hexose; HexN, hexosamine; HexNA, hexosaminuronic acid; Hep, heptose; IMS, ion mobility spectrometry; HF, hydrogen fluoride; Kdo, 2-keto-3-deoxyoctulosonic acid; LOS, lipooligosaccharide; LPS, lipopolysaccharide; NeuAc, *N*-acetylneuraminic acid; OS, oligosaccharide; P, phosphate; PBMCs, peripheral blood mononuclear cells; PEA, phosphoethanolamine; TLR4, Toll-like receptor 4.

ABSTRACT

The pathogenicity of *Campylobacter concisus*—increasingly found in the human gastrointestinal (GI) tract—is unclear: Some studies indicate that its role in GI conditions has been underestimated, whereas others suggest that the organism has a commensal-like phenotype. For the enteropathogen *C. jejuni*, the lipooligosaccharide (LOS) is a main driver of virulence. Here, we investigated the LOS structure of four *C. concisus* clinical isolates and correlated inflammatory potential of each isolate with bacterial virulence. Mass spectrometric analyses of lipid A revealed a novel hexaacylated diglucosamine moiety with two or three phosphoryl substituents. Molecular and fragment ion analysis indicated that the oligosaccharide portion of the LOS had only a single phosphate and lacked phosphoethanolamine and sialic acid substitution, which are hallmarks of the *C. jejuni* LOS. Consistent with our structural findings, *C. concisus* LOS and live bacteria induced less TNF-α secretion in human monocytes than did *C. jejuni*. Furthermore, the *C. concisus* bacteria were less virulent than *C. jejuni* in a *Galleria mellonella* infection model. The correlation of the novel lipid A structure, decreased phosphorylation, and lack of sialylation along with reduced inflammatory potential and virulence support the significance of the LOS as a determinant in the relative pathogenicity of *C. concisus*.

Key words: *Campylobacter concisus*; cytokines; glycolipids; inflammation; lipid A; lipooligosaccharide; mass spectrometry; monocytes; phosphorylation; toll like receptors

INTRODUCTION

Campylobacter concisus is a member of the *Campylobacter* genus with as yet unclear pathogenic potential. Following the initial identification of *C. concisus* in patients with periodontal disease, recent investigations indicate this non-*jejuni*/*coli Campylobacter* species has a role in acute and chronic human gastrointestinal (GI) conditions (1–5). This notion however, remains a topic of debate as in general its overall high prevalence in patients and healthy individuals alike suggests a commensal-like nature for *C. concisus*, when compared to *C. jejuni* (6, 7). Nonetheless, increased prevalence of *C. concisus* DNA in samples of patients with ulcerative colitis, pediatric Crohn's, gastroesophageal reflux and Barrett's esophagus disease have been reported (5, 8–12). Furthermore, *C. concisus* and other non-*jejuni*/*coli Campylobacter* species are implicated as casual agents in prolonged mild endemic diarrhea in children and in traveller's diarrhea (11, 13, 14) and importantly, recent meta-analyses revealed an association of *Campylobacter spp.,* mainly *C. concisus* and *C. showae*, with increased risk of inflammatory bowel disease (15).

C. concisus can attach to and invade intestinal epithelial cells, and can cause the secretion of pro-inflammatory cytokines, likely by a Toll-like receptor 4 (TLR4)-dependent mechanism (9, 16). Adherence, invasion and pro-inflammatory phenotype may be strain-specific characteristics important in the pathogenesis of Proteobacteria such as *C. concisus* and some *Escherichia coli* (17). In general, bacterial lipopolysaccharide (LPS) and lipooligosaccharide (LOS) activate TLR4 and are important virulence factors for Gram-negative bacteria (18–21). In *C. jejuni,* the hydrophobic lipid A backbone of the LOS is hexaacylated and is known to be a potent activator of TLR4 (22, 23). The interaction of LOS with TLR4 triggers a downstream signaling cascade by activation of the NF-κB transcription factor and subsequent secretion of pro-inflammatory cytokines such as TNF-α and IL-8. Apart from TLR4 engagement, *C. jejuni* LOS also plays a role in bacterial invasion, colonization and stress survival (23–25).

The biosynthesis region of *C. jejuni* LOS is highly variable between species possessing major differences in the content and organization of genes encoding LOS carbohydrate moieties and their

linkages. Additionally, the LOS gene locus is prone to frequent phase variations, resulting in broad structural heterogeneity among strains (26, 27). Major differences are observed in the outer region of the oligosaccharide (OS) moiety, but variations in lipid A phosphorylation and number of amide linkages are also present (22). The lipid A disaccharide of *C. jejuni* is comprised of either 2-amino-2-deoxy-D-glucose (glucosamine, GlcN) or 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N), thus, the number of amide linkages to the acyl chains can vary from two (GlcN-GlcN) up to four (GlcN3N-GlcN3N). The lipid A is hexaacylated with either myristic (C14:O) or palmitic (C16:O) fatty acids, four of which are hydroxy-fatty acids and are directly linked to the disaccharide residue (22, 28).

There is significant heterogeneity in the position and number of phosphate (P) and phosphoethanolamine (PEA) residues bound to the OS and lipid A of the *C. jejuni* LOS (28). Amide linkages and lipid A phosphorylation have been shown to impact TLR4 activation as reported by us and others (22, 29, 30). While the core OS of *C. jejuni* is primarily conserved, significant variations in the outer OS region (31), which can contain *N*-acetylneuraminic acid (NeuAc, sialic acid) residues that are thought to modulate TLR4 activation, have been found (22, 32, 33).

Although there has been progress in the understanding of *C. jejuni* LOS and its role in the pathogenesis of human disease (22, 32, 34), the structure and heterogeneity of *C. concisus* LOS has remained unstudied. Such investigations could be informative regarding the relative pathogenicity and/or commensalism of *C. concisus* in humans. The aims of this study were to delineate characteristics of the *C. concisus* LOS structure and inflammatory potential in comparison to the characteristics of *C. jejuni* LOS.

MATERIALS AND METHODS

Bacterial strains and LOS extraction

Clinical *C. concisus* isolates were isolated from the sigmoid colon biopsy of a newly-presenting male pediatric patient diagnosed with Crohn's disease as part of the BISCUIT study (B38) (35) and from feces of patients with acute gastroenteritis (2010-131105, 2010-347972) (10, 11). *C. concisus* NCTC 12408 strain is commercially available and originates from pediatric enteritis. *C. jejuni* 11168H is a variant of the human diarrhea isolate NCTC 11168 (36). The latter two strains were a kind gift from David Guilliano, University of East London, UK and Brendan Wren, London School of Hygiene and Tropical Medicine, London, UK, respectively. Bacterial strains were grown on blood agar No. 2 containing 0.5% yeast extract (both, Oxoid, Basingstoke, UK) and 5% defibrinated horse blood (Sigma-Aldrich, Gillingham, UK). All strains were grown at 37°C in a gas jar under microaerobic conditions generated by using a Campygen sachet (Oxoid). For *C. concisus* strains the microaerobic atmosphere was supplemented with ~10% H_2 generated with sodium borohydride (Sigma-Aldrich) (37). *C. jejuni* and *C. concisus* strains were grown for 24 h and 72 h, respectively, harvested, and stored at -80°C prior to use. Bacterial LOS was extracted and purified by a modification of the hot phenol-water method as described previously (22).

SDS-PAGE analysis

LOS samples of 10 µg were subjected to SDS-PAGE using a 12% polyacrylamide gel (Protean II xi cell, Bio-Rad, Hercules, CA) in Tris-glycine running buffer. The gel was fixed for 1 h in 40% methanol, 5% acetic acid, and LOS was visualized by silver staining (38).

Preparation of intact LOS for MALDI-TOF MS

LOS samples were prepared for MS analysis as previously described (18). Briefly, purified LOS (10 mg/ml) was suspended in a methanol-water (1:3) solution containing 5 mM EDTA. An aliquot was desalted with cation exchange beads (Dowex 50WX8-200). The desalted sample solution was mixed

with 100 mM dibasic ammonium citrate (9:1, vol/vol) and 1-2 µl was spotted onto a thin layer of matrix composed of a 4:1 solution of 2,4,6-trihydroxyacetophenone (200 mg/ml in methanol; Sigma-Aldrich) with nitrocellulose (15 mg/ml in acetone-isopropanol (1:1); Bio-Rad). Samples were left to air dry prior to analysis.

O-deacylation of LOS

Native LOS (~300 µg) was incubated with 200 µl anhydrous hydrazine (Sigma-Aldrich) at 37°C for 2 h with intermittent vortexing. The reaction was stopped with 1 ml pre-cooled acetone (-20°C) and the *O*-deacylated LOS samples were pelleted by centrifugation at 10,000 x *g*, washed with chilled acetone, centrifuged again, dissolved in 20 µl of water, lyophilized and stored at -80°C.

Hydrogen fluoride (HF)-treatment of LOS

Phosphoesters were partially removed by HF treatment. Native LOS (10 mg/ml) was reacted with 48% aqueous HF at 4°C for 16-20 h. Excess HF was removed using a Savant SpeedVac (Thermo Fisher Scientific, Waltham, MA) with an in-line trap.

High resolution MALDI-TOF and IMS-MS

MALDI-TOF MS and IMS-MS analyses were performed on a Synapt G2 high definition MS (HDMS) system (Waters Corporation, Milford, MA) in 'sensitivity mode'. The instrument is equipped with a T-wave ion mobility cell (Triwave^{TM}) (39, 40) and was operated in MALDI mode as previously described (41). Spectra were obtained in negative- or positive-ion mode operating a neodymium-doped yttrium aluminium garnet (Nd:YAG) laser at 355 nm and 200 Hz. In general, spectra were acquired for ~1-2 min, with a scan duration of 1.0 s and an overall cycle time of 1.024 s. The instrument was calibrated using the masses of the monoisotopic ions for porcine renin substrate, intact bovine insulin and B-chain.

The T-wave device on the Synapt G2 HDMS consists of three cells; a Trap cell, IMS cell, and Transfer cell. For IMS-MS experiments, the T-wave peak height voltage was 40 V and the T-wave

velocity used was generally a variable wave velocity of 650 to 250 m/s. Typically, the T-wave mobility cell contained nitrogen at a pressure of ~2 mbar, trap gas flow was 0.4 ml/min, helium cell gas flow was 180 ml/min, IMS gas flow was 90 ml/min, and trap DC bias was 80 V.

Tandem mass spectrometry (MS/MS) with IMS was performed typically by selection of precursor ions with instrument LM and HM resolution settings of 4.7 and 15.0, respectively. In initial TOF MS/MS mode without IMS, fragmentation was achieved by applying collision energy with argon as the collision gas in the Trap region of the T-wave ion mobility cell. Collision energies of 85-110 V were required for optimum fragmentation of intact LOS or of prompt fragment ions (pseudo-MS^3) in the Trap. For IMS-MS/MS experiments, collision energy was applied also with argon as the collision gas in the Transfer cell following IMS separation, at values ranging from 50-90 V, depending on the analyte. For analysis of OS and lipid A prompt fragments, a T-wave variable wave velocity of 1100 to 200 m/s was used. Two-dimensional IMS spectra were viewed using DriftScope 2.1 software, and selected spectral regions were exported to MassLynx with retention of drift time information for generation of mobilograms and subspectra. Chemical structures were generated using the ChemBioDraw Ultra software.

Genomic analysis

Genomic analysis of the *C. concisus* strains used in our study was carried out to look for the presence or absence of genes involved in PEA transfer and in sialic acid biosynthesis and transfer. The genomes of three of the strains (B38, 2010-131105, 2010-347972), and 53 additional *C. concisus* strains were recently whole genome sequenced and genomes assembled (42). In addition, the following analyses were carried out on 36 other publicly available *C. concisus* (2, 43–47).

Gene and protein prediction of genome assemblies was carried out with Prokka (48). The PROKKA dependencies BioPerl (49), GNU Parallel (50), BLAST+ (51) and Prodigal (52) were utilized along with the recommended and optional tools Aragorn (53), Barrnap (https://github.com/Victorian-Bioinformatics-Consortium/barrnap ; 42), HMMER3 (54), Infernal (55), RNAmmer (56), and SignalP (57). Annotation of predicted genes and proteins was carried out with BlastKOALA (58).

PCR validation of LOS genes

PCR validation of the genome analysis was performed using the primer pairs *eptC*-F (CCAGATGAAGCCGGTGAGTT) and *eptC*-R (T[G/A]CTCCAAGGCT[C/T]TTTGCTT), which amplifies a 578 bp region of the PEA transferase gene *eptC*, and the primer pair *waaC*-F (TGGCT[A/C]GTTGATGCCCGTTT) and *waaC*-R (ATCGCCTCAGCTCTTGC[T/C]TT), which amplifies a 522 bp region of the LOS heptosyl transferase I *waaC* gene which is the first within the LOS gene cluster. PCR conditions were as follows: *eptC* - 94°C for 5 min, 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. *waaC* - 94°C for 5 min, 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min.

TNF-α secretion by THP-1 monocytic cells and PBMCs

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA), and propagated in RPMI 1640 supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere. The cells were differentiated with 10 ng/ml PMA (Sigma-Aldrich) for 18 h as previously reported (59). The differentiated THP-1 cells were seeded at 1.2 x 10^5 cells per well in 96-well plates and treated with LOS (100 ng/ml) or culture media only for 18 hours. The supernatants were collected and stored at -80°C until analysis.

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood obtained from seven healthy adult volunteers following the manufacturer's protocol (Lymphoprep™; Axis-Shield, Dundee, UK). PBMCs (2 x 10^6 cells/ml) were suspended in RPMI 1640 medium with 10% FBS and co-cultured with bacteria (multiplicity of infection = 100) and supernatants were collected after 18 h. TNF-α cytokine release were determined using a TNF-α ELISA kit (Ready-Set-Go kit, eBioscience/Affymetrix, San Diego, CA) following the manufacturer's instructions.

Blood samples were obtained with informed consent and ethical approval from the Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee and in accordance with the Declaration of Helsinki.

Galleria mellonella infection model

G. mellonella larvae (UK Waxworms Ltd., Dinnington, UK) were injected into the second foreleg with a bacterial suspension in 10 µl inocula (n=15, ~10^7 CFU) (60, 61). Larvae were incubated at 37°C and survival was monitored at 24 h intervals for 5 days. PBS injection served as control.

Statistical analysis

One-way ANOVA with Tukey post-test was applied for multiple comparison analysis. Survival curves were analyzed by Mantel-Cox log-rank test. GraphPad Prism 7.00 software was used for analysis.

RESULTS

Heterogeneity between *C. concisus* LOS structures

To investigate the structure of *C. concisus* LOS, we first analyzed intact LOS that was isolated from four *C. concisus* GI strains by MS and SDS-PAGE. LOS was also extracted from the *C. jejuni* 11168H strain and used for comparison. Spectra of the intact LOS were obtained in high-resolution reflectron mode negative-ion MALDI-TOF MS that enabled measuring monoisotopic molecular ions with high mass accuracy (<35 ppm). The spectra provided evidence of varied and multiple LOS molecular ions with some ions detected above *m/z* 5000 in all four *C. concisus* LOS samples (**Fig. 1A-D**). Peaks for the molecular ions of the *C. jejuni* 11168H LOS were of lower mass in the range of *m/z* 3600-4300 (Fig. 1E). This is in agreement with previous MS studies of intact *C. jejuni* LOS from other strains which reported molecular ion peaks of lower mass than those for the LOS of the *C. concisus* isolates (22, 62, 63).

The SDS-PAGE profile of the *Campylobacter* LOS showed the presence of multiple bands in all samples (Fig. 1F), which was consistent with the heterogeneity in the LOS that was revealed by the MS analyses. In accordance with the MALDI-TOF MS data, the electrophoretic mobility of the slow migrating bands was indicative of larger LOS moieties in the *C. concisus* isolates compared to the *C. jejuni* LOS. The *C. concisus* isolates with bands for the largest LOS moieties were B38 and 2010-131105, and these appear to be in accord with the molecular ion peaks observed in the negative-ion spectra at *m/z* 5598.18 (Fig. 1B) and *m/z* 5413.32 (Fig. 1C), respectively.

Prompt fragmentation of the labile 2-keto-3-deoxyoctulosonic acid (Kdo)-lipid A bond of the LOS occurring in the MALDI source produced abundant lipid A and OS fragment ions of the LOS. Observed peaks corresponding to the loss of Kdo (220.06 Da) aided in identification of peaks for OS fragment ions and LOS molecular ions. An attribute of this methodology is that abundant lipid A and OS fragment ions are produced together with molecular ions for the intact LOS that, thus, enables more certain identification of the latter and provides an opportunity to obtain top-down sequence information. For example, the observed peak at *m/z* 1904.23 for the monoisotopic mass of the largest lipid A fragment ion

of *C. concisus* isolate 2010-131105 LOS (Fig. 1C) together with that for the OS fragment ion at *m/z* 2287.59 plus a proton gives a value of *m/z* 4192.83, corresponding to the monoisotopic mass of the prominent molecular ion peak for intact LOS.

Ion mobility spectrometry (IMS) MALDI-TOF MS was used for the separation of intact LOS and the prompt lipid A and OS fragment ions formed in the source (**Fig. 2**). Due to the differences in the shape and size of the lipid A and OS fragments and intact LOS, the ions were separated by IMS to provide more evidence of distinct molecular characteristics. In some cases, the ion types occurred in otherwise overlapping regions of the spectra. The IMS enabled extraction of subspectra of specific ion types, permitting unambiguous classification of ions and mass measurements. The spectra of the LOS from each isolate contained unique OS fragment ions and molecular ions for intact LOS but contained some similar peaks for lipid A fragment ions (Table 1).

Tandem MS analysis of the lipid A moiety

We performed negative-ion tandem (MS/MS) analysis with IMS to obtain sequence information and structural details regarding the lipid A and OS moieties (**Fig. S1**). Differences in the IMS drift time of the lipid A fragment ions compared to ions for OS fragments or non-LOS species of similar *m/z* values resulted in cleaner MS/MS spectra and enabled more confident interpretations of the MS/MS spectra. This is illustrated by the MS/MS spectrum of the lipid A fragment ions at *m/z* 1824.3 (Fig. S1B, top) from *C. concisus* LOS strain B38. Without IMS, we would have detected some of the less abundant, but significant non-lipid A fragment ions deriving from the peak at *m/z* 1821.60 shown in the MS/MS spectrum (Fig. S1B, bottom).

Hydrazine treatment of LOS was performed to hydrolyze the *O*-linked acyl chains from the lipid A while the *N*-linked fatty acyl groups remained intact. An IMS-MS/MS negative ion spectrum (**Fig. 3A, C**) was obtained of the *O*-deacylated lipid A fragment ion (*m/z* 979.5) from the LOS of *C. concisus* strain 2010-131105. The fragment ion peaks observed were in accordance with the presence of a disaccharide containing two GlcN residues with β-hydroxy myristic acid (C14:0(3-OH)) and β-hydroxy palmitic acid

(C:16(3-OH)) linked to the amino groups on the non-reducing and reducing terminal monosaccharide residues, respectively. Peaks corresponding to B- and Y-type fragment ions as well as cross-ring fragment ions (^{0,2}A and ^{0,4}A) provided evidence for two different lipid A structures (Fig. 3A) – one with phosphate on both ends (bis-phosphorylated) and one di-phosphorylated lipid A with a single pyrophosphoryl group (Table S1).

Negative-ion IMS-MS/MS of the *m/z* 1824.3 ions for intact lipid A of strain 2010-131105 produced fragment ion peaks at *m/z* 1726.33 corresponding to loss of H_3PO_4, (-97.98 Da), at *m/z* 1580.10 consistent with loss of C14:0(3-OH) (-244.20 Da), and at *m/z* 1281.96, consistent with sequential losses of H_3PO_4, C14:0(3-OH) and C12:0 (-200.18 Da). The ^{0,4}A-type cross-ring, B- and Y-type fragment ions detected in the MS/MS spectrum of the intact lipid A also were in accord with the expression of di- and bis-phosphorylated hexaacylated lipid A (**Fig. 3B, D**) confirming our interpretation of the MS/MS spectrum of the *O*-deacylated lipid A. In addition, low mass fragment ions were observed in the negative-ion MS/MS spectra at *m/z* 158.9 for $HP_2O_6^-$, providing further support for the presence of pyrophosphoryl groups on the diphosphorylated lipid A.

In total, we identified five different lipid A moieties in *C. concisus* LOS (Table 2). All were in accordance with a disaccharide of two GlcN residues that contained a total of two amide linkages. Fragment ions at *m/z* 1824.2 or higher corresponded to the same fatty acid profile as the lower mass fragment ions at *m/z* 1796.2, but with exchange of a C16:0(3-OH) residue for a C14:0(3-OH). An additional phosphate or PEA moiety accounted for all of the other high mass lipid A species observed in the spectra of the *C. concisus* isolates (Table 2).

Overall, the data showed that the hexaacylated *C. concisus* LOS has some similarity to *C. jejuni* LOS, however clear differences were identified (**Fig. 4**). Both contain four hydroxy fatty acids, but there is a single C16:0(3-OH) in the majority of *C. concisus* lipid A moieties, in place of one of the C14:0(3-OH) moieties on *C. jejuni* LOS. There are two secondary fatty acids on the non-reducing terminal glucosamine in lipid A of both species, but these were identified as C14:0 and C12:0 in *C. concisus*, in contrast to the two C16:0 acyl chains on the non-reducing terminal glucosamine of *C. jejuni* lipid A (28, 64).

Analyses of the OS moieties

While the lipid A moieties of the *C. concisus* strains possessed significant similarities, we identified peaks of unique masses corresponding to OS fragment ions in the spectra of the LOS of each isolate (Fig. 1, Table 1). HF treatment of the LOS was used to remove and aid in establishing the presence of phosphoesters. Comparison of positive-ion MALDI-TOF MS spectra of the HF-treated and untreated LOS of *C. concisus* NCTC 12408 confirmed that there was a single phosphate substituent (HPO_3, 79.97 Da) as part of the inner core OS observed at *m/z* 2108.6 in untreated LOS (**Fig. S2**).

The presence of a single phosphate in the core OS was further established by MALDI-TOF IMS MS/MS analyses in the positive-ion mode of the untreated and HF-treated NCTC 12408 LOS. Extensive fragmentation produced losses in accord with the presence of expected monosaccharide components such as Kdo, heptose (Hep; 192.06 Da), hexose (Hex; 162.05 Da) and also hexosamine (HexN; 161.07 Da) (**Fig. S3**). A loss of 175 Da, possibly corresponding to hexosaminuronic acid (HexNA; 175.05 Da) was detected in some of the spectra and apparently corresponded to low mass fragment ion peaks observed in positive-ion spectra at *m/z* 176.06 as observed in Fig. S3.

Elements of commonality were detected in the spectra of the LOS from *C. concisus*. Monoisotopic OS fragment ions were observed at *m/z* 2106.56 in the negative-ion spectra of intact LOS from both NCTC 12408 and 2010-347972 (Fig. 1A, D) presumably for the core OS. In addition, there was a neutral loss of 1220.4 Da from both LOS molecular ion and OS fragment ions in spectra of NCTC 12408 (OS peaks at *m/z* 3326.92 and 2106.56) and 2010-131105 (OS peaks at *m/z* 3508.09 and *m/z* 2287.59; Fig. 1A, C). The OS structures could be related to semi-rough type LPS observed in some Gram-negative bacteria that contain one *O*-antigen repeat unit (65).

Lack of non-reducing terminal sialic acid (NeuAc) on the *C. concisus* LOS was established by negative-ion MS/MS of molecular ions of intact LOS (**Fig. 5**). Collisional activation of the molecular ions of the intact LOS from the four strains of *C. concisus* (Fig. 5A-D) produced abundant fragment ions for loss of Kdo (220.06 Da), followed by loss of HPO_3, and loss of H_2PO_4 (97.98 Da). For example, MS/MS of $(M-H)^-$ of intact LOS from NCTC 12408 at *m/z* 5152.10 produced a prominent peak at m/z 4932.02 for

loss of Kdo (Fig. 5A). Similarly, collisional activation of the $(M-H)^-$ ions at *m/z* 4226.87 for intact LOS from strain B38 produced a prominent peak at *m/z* 4006.82 corresponding to loss of Kdo, and also a peak at *m/z* 3926.82 for loss of both Kdo and HPO_3 (Fig. 5B). Peaks for similar fragments of the LOS molecular ion were also observed for the other two *C. concisus* strains (Fig. 5C, D).

In contrast, the MS/MS spectra of molecular ions of the intact LOS from *C. jejuni* (Fig. 5E-F) contained fragment ion peaks consistent with loss of Kdo as well as loss of both, Kdo and NeuAc (291.10 Da). For example, collisional activation of the $(M-H)^-$ ions at *m/z* 4292.08 (Fig. 5E) from the *C. jejuni* LOS produced abundant fragment ions at m/z 4072.00 for loss of Kdo, and peak at *m/z* 3780.93 for loss of Kdo and NeuAc. Fragment ions from loss of NeuAc are consistent with non-reducing terminal sialylation that we have previously established as present on the 11168H LOS (22). However, corresponding fragment ion peaks for the loss of NeuAc were strikingly absent in the MS/MS of intact LOS from the 4 *C. concisus* strains.

Genomic analyses of LOS biosynthetic genes

We conducted a genomic analysis of three *C. concisus* strains used in our study to look for the presence or absence of genes involved in PEA transfer and in sialic acid biosynthesis and transfer. We looked specifically for the presence of sialic acid synthase (*neuB1*), sialic acid transferase (*cstII*), PEA transferase (*eptC*) along with heptosyl transferase (*waaC*) which is known to be the first gene within the LOS gene cluster (66). Analysis of whole genome sequences indicated that all three strains contained *waaC*, whereas none of the strains contained *neuB1* or *cstII* confirming the lack of sialic acid synthesis machinery. Presence of *waaC* was further confirmed by PCR analysis. This lack of sialic acid synthesis capability was also confirmed in all 89 published *C. concisus* genomes. When we looked for the presence of the PEA transferase *eptC* gene within all of the *C. concisus* genomes, we found that most strains (69%) did not contain the *eptC* gene, however all three of the strains used in this study were *eptC* positive which was also confirmed by PCR analysis.

Inflammatory and virulence potential of *C. concisus* isolates

The LOS-induced TLR4 signaling cascade is crucial in triggering monocytic innate immune responses to *C. jejuni* that result in TNF-α secretion (22). To determine the pro-inflammatory potential of *C. concisus* LOS, differentiated THP-1 monocytes were treated with purified LOS from all 4 *C. concisus* strains and from the *C. jejuni* 11168H strain for 18 h prior to quantification of the pro-inflammatory cytokine, TNF-α. Notably, the LOS from all *C. concisus* strains elicited a significantly reduced TNF-α response when compared to the *C. jejuni* 11168H LOS (**Fig. 6A**; $p<0.001$). To confirm the importance of LOS signaling in this context, PBMCs from seven healthy donors were co-cultured with live bacteria of each of the four *C. concisus* strains and *C. jejuni*. As seen in the response to purified LOS, the *C. concisus* live bacteria also induced significantly less TNF-α when compared to *C. jejuni* 11168H (Fig. 6B; $p<0.05$).

To assess bacterial virulence, we used a *Galleria mellonella* infection model. Larvae were infected with *C. concisus* isolates and *C. jejuni* 11168H and survival was monitored over five days. *C. concisus* isolates were significantly less virulent (>85% *G. mellonella* survival) when compared to *C. jejuni* (36% survival) (Fig. 6C; $p<0.001$). Thus, our findings show that the *C. concisus* is less inflammatory and less virulent compared to *C. jejuni* and, furthermore, that these differences are reflected by the reduced inflammatory potential of the *C. concisus* LOS when compared to the LOS of *C. jejuni*.

DISCUSSION

C. concisus is being increasingly found in the human GI tract, yet the contribution of this organism to homeostasis in health or to disease states is unclear. In this study, we aimed at defining and correlating the structure of *C. concisus* LOS with its bioactivity. Four clinical isolates were chosen from adult and pediatric patients presenting with gastroenteritis and Crohn's disease. The intact LOS of all four *C. concisus* isolates was larger than that of *C. jejuni*, which was primarily due to differential size of the OS moiety. In addition, significant heterogeneity between the OS of the *C. concisus* isolates was observed. Heterogeneity in the *C. jejuni* LOS, particular in the outer region of the OS, has been previously described and was ascribed to differences in the gene content and frequent phase variations in the LOS gene loci (26). Variations primarily were observed in the outer region of the OS while core OS and the general structure of the lipid A were found to be predominantly conserved (31). In line with these findings, we observed structural similarities in the lipid A and in likely monosaccharide components of the core OS in all four *C. concisus* isolates while there appeared to be greater variability in the outer OS regions.

Hexaacylation has been found to be a requisite for optimal engagement with TLR4 and its downstream signaling cascade (22). Despite the presence of a hexaacylated lipid A moiety in LOS of both *C. concisus* and *C. jejuni*, we observed less induction of TNF-α by PBMCs in response to *C. concisus* strains compared to *C. jejuni.* This may be due to the recognized relationship between the three-dimensional shape of lipid A and its bioactivity. Specifically, it has been found that lipid A molecules with a more conical wedge-shaped conformation and a hydrophilic backbone that is smaller compared to that of the hydrophobic portion of the molecule as found in *C. jejuni* induce more inflammatory signaling compared to molecules with a more cylindrical shape as would be expected for the *C. concisus* lipid A due to the lack of secondary C16 acyl groups (67). Moreover, although *C. jejuni* strains can have up to two GlcN3N moieties in the lipid A, our data showed that the lipid A of the four *C. concisus* isolates contained no GlcN3N moieties, which is thought to impact on recognition of lipid A by the TLR4-MD2 complex due to differences in the flexibility of amide- versus ester-linked acyl chains (22, 30).

Apart from the nature of the acylation of the lipid A, other modifications of LOS/LPS have been found to influence engagement with TLR4 and inflammatory signaling (20). Based on analysis of a panel of livestock and non-livestock associated *C. jejuni* isolates, we previously reported that increased numbers of phosphoryl substituents on the lipid A, and the presence of sialic acid in the OS contributed to TLR4-mediated inflammatory signaling by THP-1 cells and primary human monocytes as reflected by TNF-α secretion (22).

C. jejuni produces LOS containing a core OS but lacking the repeating *O*-antigens, although *C. jejuni* LPS has been described (68, 69). Indeed, as reported by Karlyshev and colleagues, all *C. jejuni* strains express LOS while the LPS structure observed in some strains appears to be genetically and structurally unrelated and bears closer resemblance to capsular polysaccharides (70). LOS of *C. jejuni* is heavily phosphorylated, containing one or two phosphate and up to three PEA groups on the lipid A and in many strains a PEA on the OS moiety (22). The presence of PEA on the cell surface of *C. jejuni* is associated with bacterial virulence by various mechanisms. Two recent studies identified the *eptC* gene encoding a novel transferase conferring PEA expression on flagella rod protein, *N*-linked glycans and lipid A (71, 72). The addition of PEA to lipid A and the Hep on the core of the OS results in enhanced recognition of TLR4, resistance to antimicrobial peptides, and facilitating the ability of *C. jejuni* to colonize and survive in the avian and murine host (29). Variations in PEA and pyrophosphorylation of lipid A also have been shown to play an important role in TLR4 signaling by the pathogenic species, *Neisseria meningitidis* and *N. gonorrhoeae,* while PEA and pyrophosphate are absent in the lipid A moiety of most commensal *Neisseria* strains that rarely cause disease and exhibit lower inflammatory potential (18, 73).

In our analyses of the *C. concisus* LOS, we found no evidence for PEA in the OS moiety but did detect an 80 Da difference corresponding to phosphate as part of the core of the OS moiety. Overall most prominent lipid A peaks were consistent with non-PEA containing lipid A, although spectra of the intact LOS from two *C. concisus* isolates, NCTC 12408 and B38, contained a major peak for a lipid A moiety containing PEA. Interestingly, *ept*C genes were encoded in all three *C. concisus* strains subjected to whole genome sequencing and this was further confirmed by PCR analysis. Despite this, two of the

isolates did not express a major peak for PEA containing lipid A suggesting that PEA expression may undergo genetic regulation. Altogether, no more than three phosphoryl substituents (including both P and PEA) were detected on any of the *C. concisus* lipid A moieties. In contrast, our previous MS analyses of the *C. jejuni* LOS revealed that the lipid A contained a total of from 2-5 phosphoryl-containing groups (22). Thus, the extent of phosphorylation and phosphoethanolaminylation of the *C. concisus* lipid A was less than that of the *C. jejuni* lipid A, raising the hypothesis that the reduced cytokine and *G. mellonella* response may at least be partly due to the observed differences in lipid A phosphorylation across this genus.

LOS sialylation is another important virulence factor in *C. jejuni* that is associated with enhanced inflammation, increased colonization, and increased risk of developing Guillain-Barré syndrome, the latter arising due to molecular mimicry between the *C. jejuni* LOS and gangliosides found in peripheral nerves (22, 25, 33, 74). In a previous study we reported the presence of up to two sialic acid residues on the OS of *C. jejuni* isolates and, importantly, increased sialylation was correlated with enhanced TLR4-mediated TNF-α secretion when compared to strains without sialic acid (22). Our MS/MS data indicate the absence of sialic acid in the LOS from the four *C. concisus* strains analyzed while confirming NeuAc as part of the reference *C. jejuni* LOS. Further, genomic analyses revealed the lack of sialic acid synthesis capability in all 89 published *C. concisus* genomes. We previously reported nonsialylated LOS produced by some environmental *C. jejuni* isolates (non-livestock associated) while all human isolates tested were sialic acid positive (22). Given the significance of sialic acid in the virulence of *C. jejuni*, the absence of sialylation of *C. concisus* LOS is intriguing as it indicates lack of an important virulence factor that also contributes to inflammatory signaling. An in-depth analysis of the existence of specific LOS biosynthesis gene loci in *C. concisus* as well as the presence of inflammation-associated genes involved in sialic acid and PEA biosynthesis and transfer is warranted to further investigate these important structural differences within the *Campylobacter* genus.

Taken together, our findings show that *C. concisus* isolates exhibit lipid A moieties and heterogeneous OS with overlapping as well as unique features compared to the well-studied *C. jejuni*

LOS. Despite many similarities in the LOS structures of these two organisms, differences in the lipid A amidation, fatty acid substituents, and phosphorylation as well as the lack of sialic acid substituents in the *C. concisus* LOS are of significance. The potential association of LOS variations with clinical outcome awaits examination of a larger sample of strains from diverse backgrounds and may give further insight into the remaining question of *C. concisus*' pathogenic potential. Further, it is interesting to speculate that these *Campylobacter* organisms occupy the same ecological niche in the human host as *C. jejuni*, which may result in inter-species cross-talk, and potential indirect effect on *Campylobacter*-related pathogenicity. Our findings on LOS composition, pro-inflammatory potential and bacterial virulence provide structural and functional insights into the *C. concisus* LOS that increase our understanding of this underexplored *Campylobacter* species and its role in human disease.

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TABLE 1. Observed $(M-H)^-$ peaks for lipid A and OS fragment ions and corresponding intact LOS

Strain	Lipid A	OS	Intact LOS
C. concisus NCTC 12408	1796.23	3326.92	5124.15
	1824.23	3326.92	5152.06
	1947.22	3326.92	5275.16
C. concisus B38	1849.27	2321.68	4171.93
	1904.20	2321.68	4226.87
			5598.18
C. concisus 2010-131105	1904.23	2287.59	4192.83
	1824.26	3508.09	5333.25
	1904.23	3508.09	5413.32
C. concisus 2010-347972	1796.22	2430.71	4227.89
	1796.22	3225.98	5023.18
	1824.26	3225.98	5051.20
C. jejuni 11168H	1878.31	1723.51	3602.82
	1878.31	1926.61	3805.91
	1878.31	2412.72	4291.94

TABLE 2. Proposed compositions and observed and calculated negative Y-ion type peaks for lipid A fragment ions of intact *C. concisus* LOS

Composition	HexN-HexN 4 C14:0(3-OH) C14:0 C12:0 2P	HexN-HexN 3 C14:0(3-OH) C16:0(3-OH) C14:0 C12:0 2P	HexN-HexN 3 C14:0(3-OH) C16:0(3-OH) C14:0 C12:0 2P PEA ($-H_3PO_4$)	HexN-HexN 3 C14:0(3-OH) C16:0(3-OH) C14:0 C12:0 3P	HexN-HexN 3 C14:0(3-OH) C16:0(3-OH) C14:0 C12:0 2P PEA
Cal. m/z	1796.21	1824.24	1849.27	1904.21	1947.25
			Obs. m/z (Δppm)		
NCTC 12408	1796.23 (10.3)	1824.23 (-7.1)			1947.22 (-16.1)
B38			1849.27 (-2.4)	1904.20 (-4.8)	
2010-131105	1796.22 (4.7)	1824.26 (9.4)		1904.23 (10.9)	
2010-347972	1796.22 (4.7)	1824.26 (9.4)			

Figure 1.

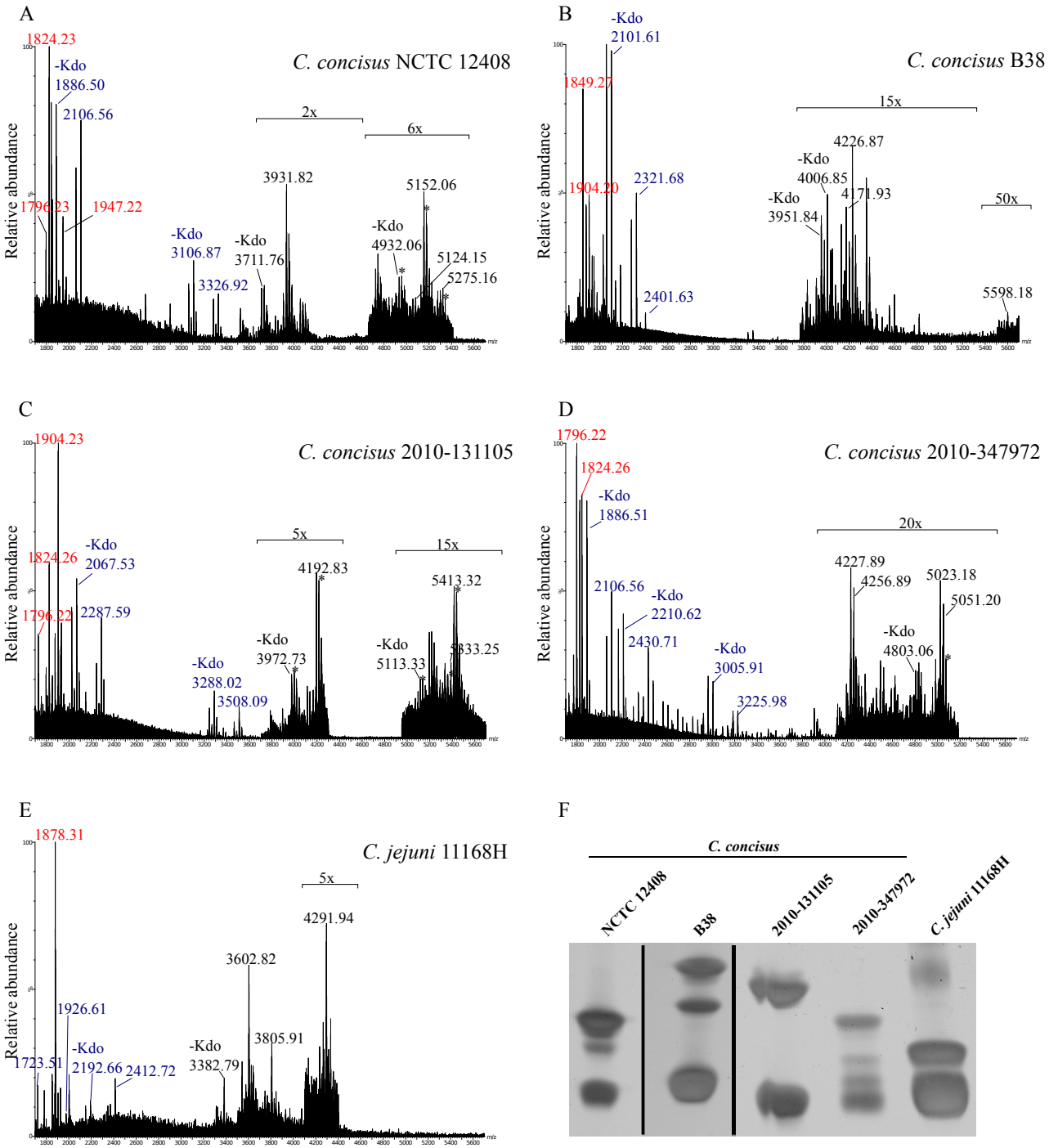

Fig. 1. Negative-ion MALDI-TOF spectra and LOS profiles of *C. concisus* and *C. jejuni* LOS.

A-E: Negative-ion MALDI-TOF MS spectra for the LOS of *C. concisus* isolates NCTC 12408, B38, 2010-131105, 2010-347972 and *C. jejuni* 11168H, respectively. Where indicated the relative abundance of the peaks was magnified. The *m/z* values presented are for the monoisotopic peaks observed, and for lipid A fragments are labeled with red-colored font, for OS fragment ions are labeled with blue-colored font and for molecular ions with black-colored font. Asterisks (*) show peaks corresponding to sodiated molecular or fragment ion peaks. Purified LOS (10 μg per lane) of *C. concisus* isolates NCTC 12408, B38, 2010-131105, 2010-347972 and *C. jejuni* 11168H were separated by SDS-PAGE and stained using silver nitrate. F: The gel was cropped where indicated (black lines).

Figure 2.

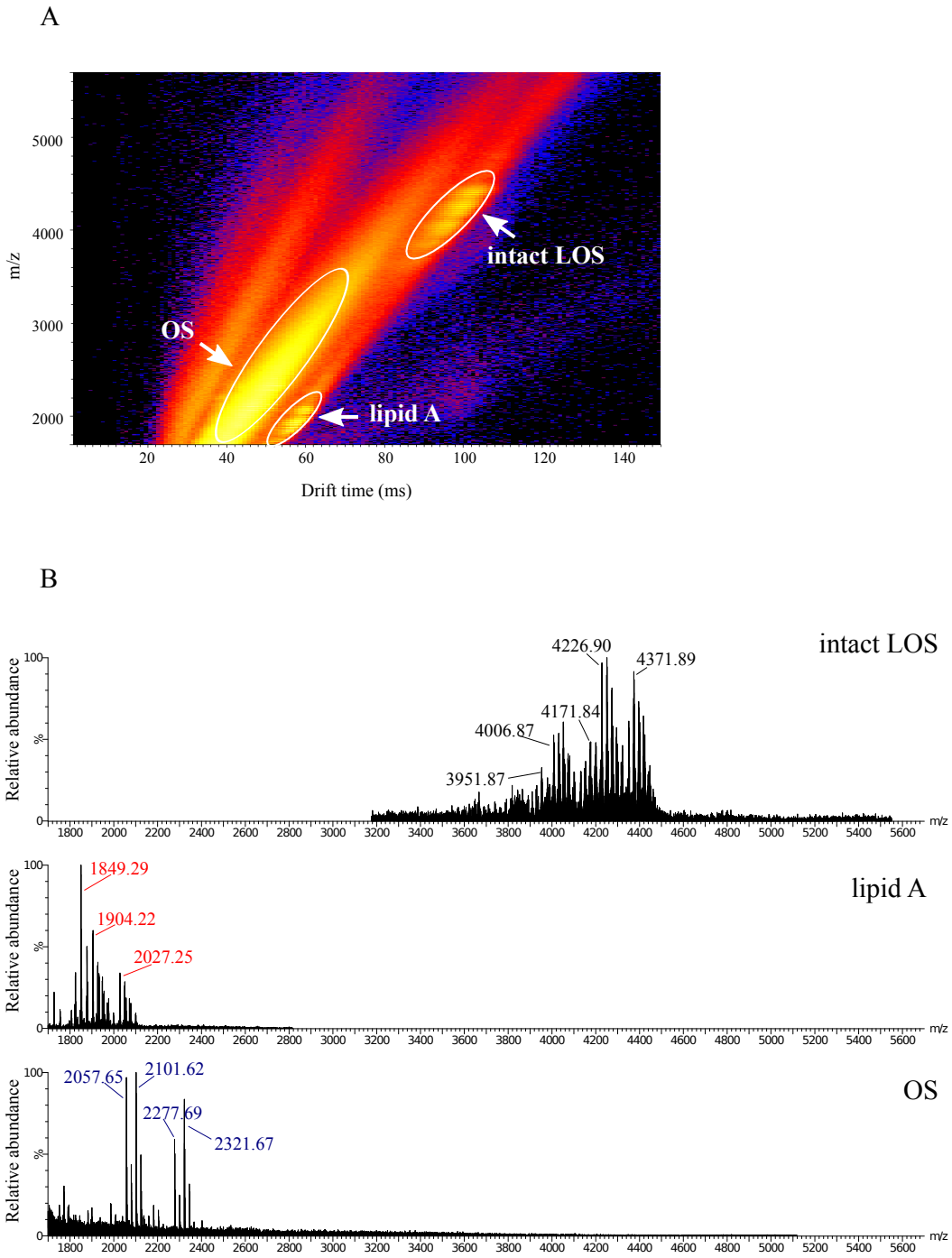

Fig. 2. Analysis of intact LOS from *C. concisus* B38 by MALDI-TOF MS with IMS.

Panel shows a two-dimensional plot of the IMS dataset. A: Differences in drift time allow for separation of low molecular weight components of non-LOS origin, OS prompt fragment ions, intact LOS, and lipid A prompt fragment ions. Regions of the mass spectra of the $(M–H)^-$ molecular ions, lipid A prompt fragments, and OS prompt fragments. B: Spectra were acquired with the Synapt G2 HDMS mass spectrometer using IMS and analyzed with DriftScope and MassLynx software.

Figure 3.

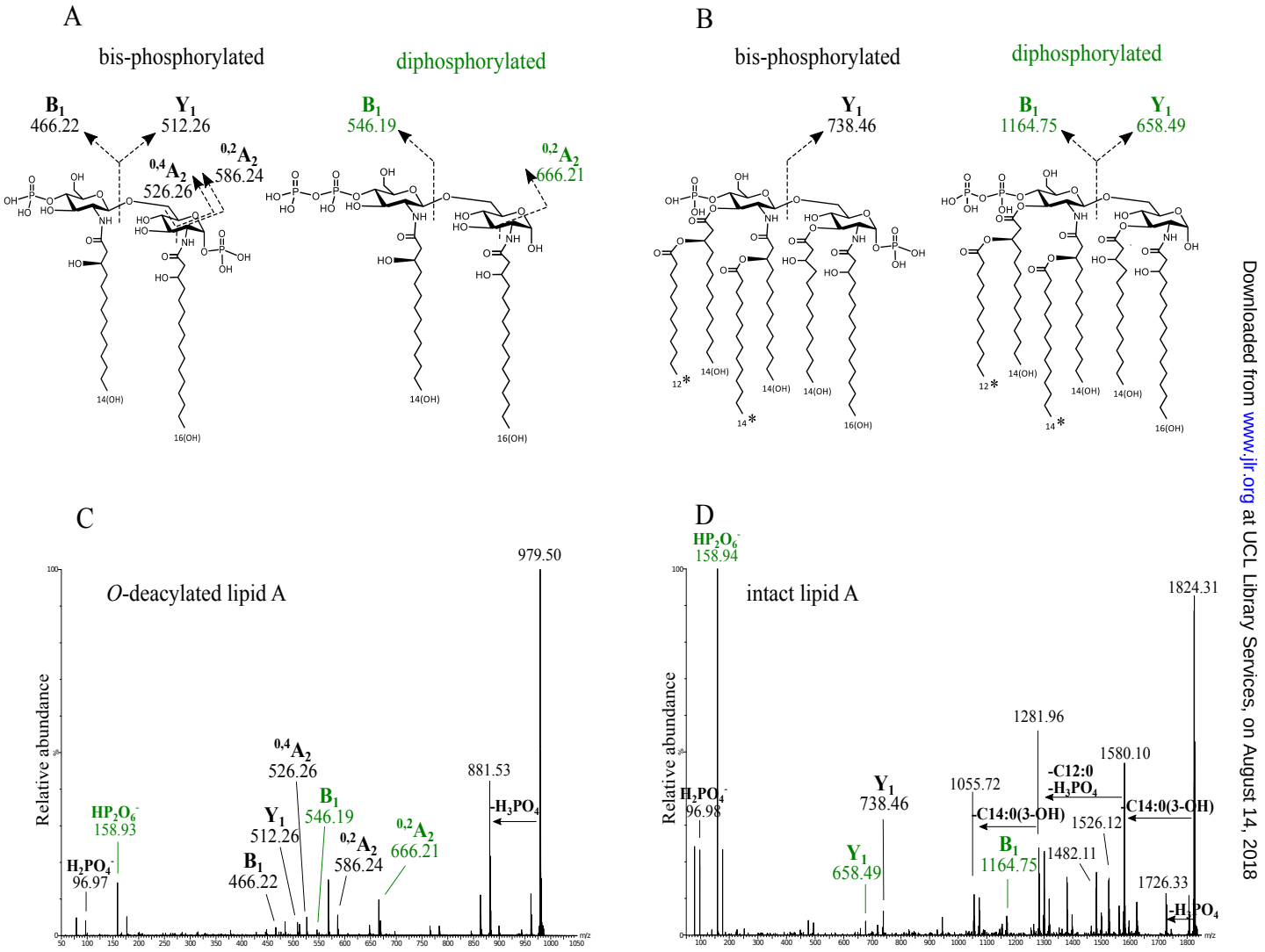

Fig. 3. Negative-ion MALDI-TOF MS/MS spectra of *O*-deacylated and intact lipid A of *C. concisus* 2010-131105.

A,C: Proposed structure and fragmentation from MS/MS of *O*-deacylated lipid A of *C. concisus* 2010-131105. B,D: Proposed structure and fragmentation from MS/MS of intact lipid A of *C. concisus* 2010-131105. Fragment ion peaks observed in the MS/MS spectrum of the parent ions at *m/z* 979.50 of the *O*-deacylated (C) and of the parent ions at *m/z* 1824.31 in the intact lipid A (D) were consistent with B-, Y-, ^{0,4}A- and ^{0,2}A-type fragment ions as indicated in the proposed structures. Asterisk (*) indicates potential interchange in the positions of two fatty acids. The green-colored labels are for fragment ions consistent with a lipid A with a single pyrophosphoryl group.

Figure 4.

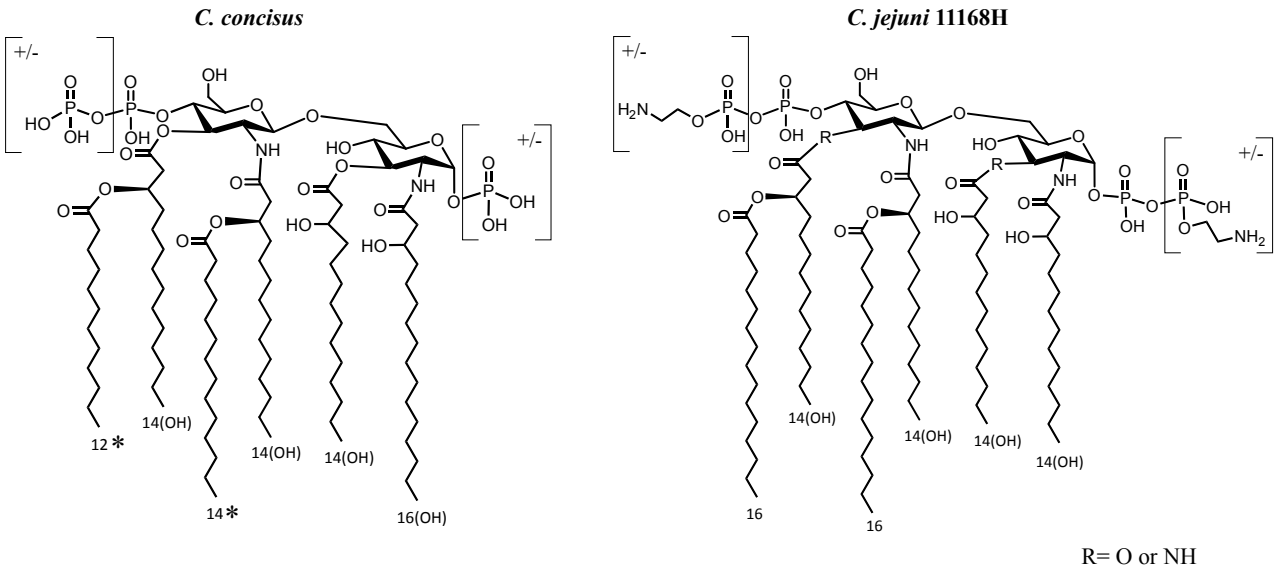

Fig. 4. Schematic of consensus structures of *C. concisus* and *C. jejuni* 11168H lipid A.

Asterisk (*) indicates potential interchange in the positions of two fatty acids.

Figure 5.

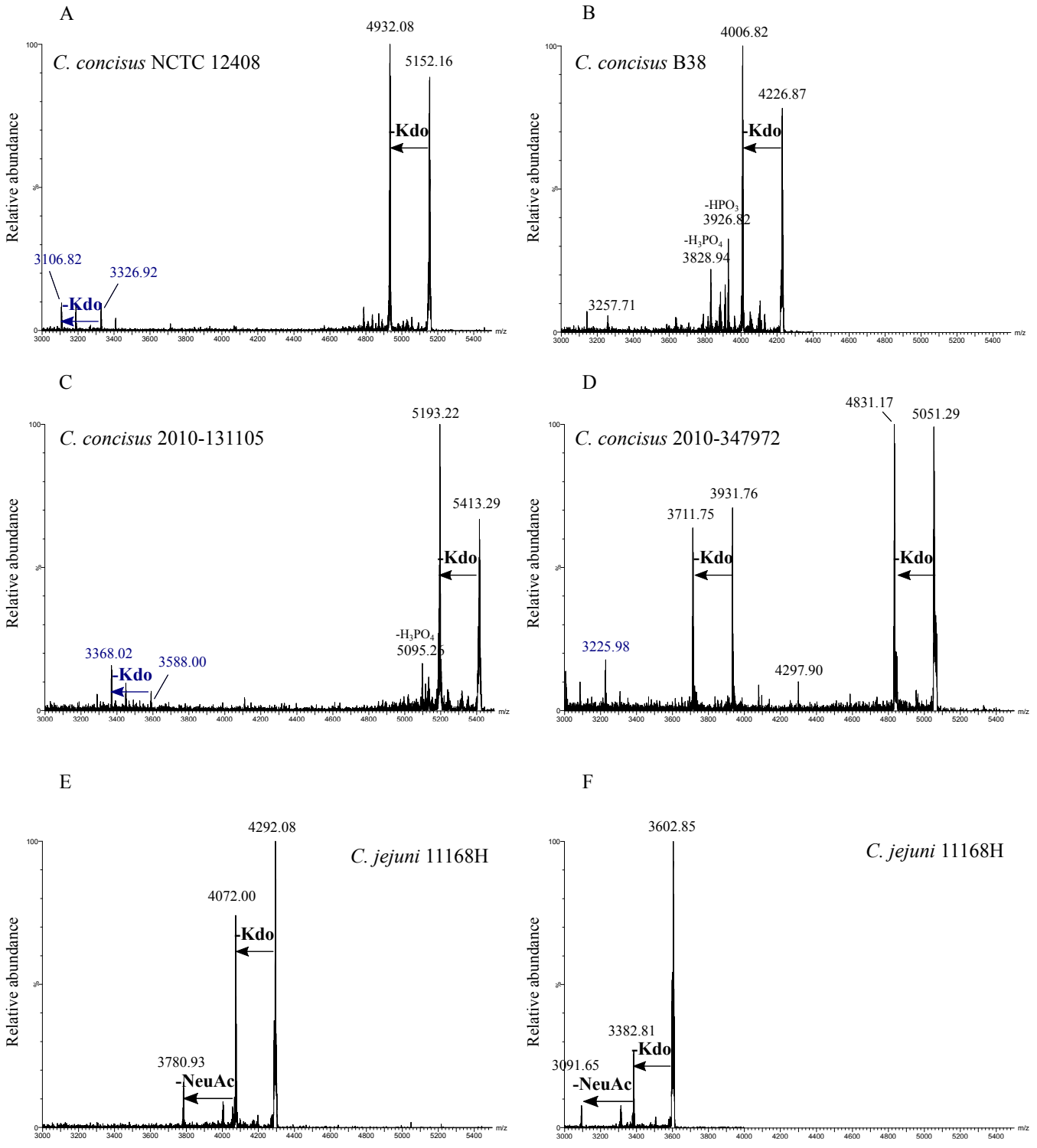

Fig. 5. Negative-ion MALDI-TOF MS/MS analysis of intact LOS ions.

A-D: Negative-ion MALDI-TOF MS/MS spectra for an LOS molecular ion $(M-H)^-$ peak of each *C. concisus* isolates NCTC 12408, B38, 2010-131105, 2010-347972. E,F: Negative-ion MALDI-TOF MS/MS spectra for two intact LOS ions of *C. jejuni* 11168H. The mass of the losses observed are consistent with the fragments of known carbohydrate or phosphoryl moieties as indicated. Labels in blue-colored font indicate fragment ions corresponding with OS fragments as previously observed.

Figure 6.

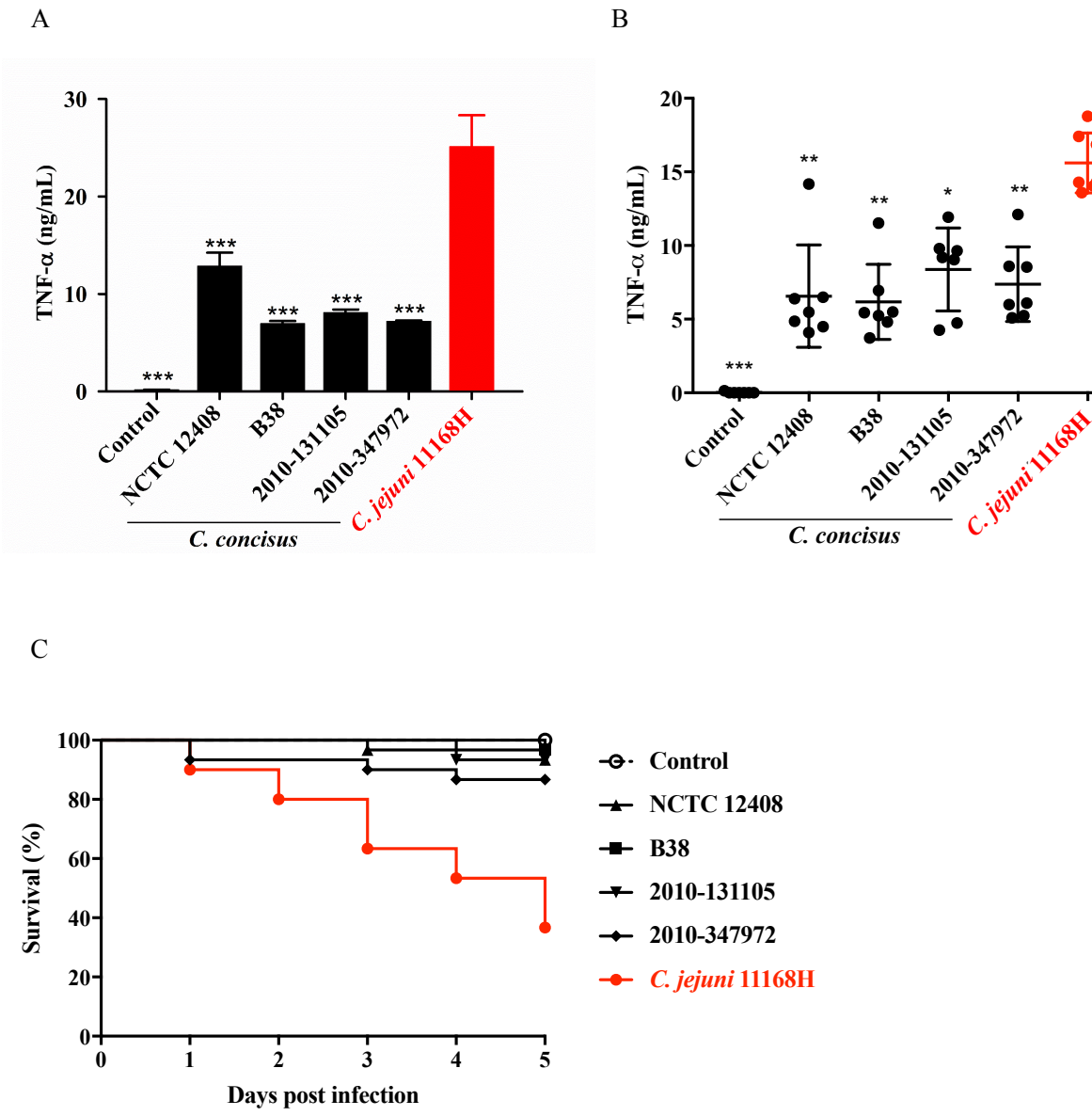

Fig. 6. Inflammatory potential and virulence of *C. concisus* isolates.

A: Differentiated THP-1 cells were stimulated with purified (100 ng/ml) LOS for 18h prior to quantification of TNF-α secretion by ELISA. Results are expressed as the mean ± SD of 8 biological replicates. Stars indicate statistical significant differences when compared to *C. jejuni* as analyzed by ANOVA with Tukey post hoc test. B: Human PBMCs (n=7) were co-cultured with live bacteria at MOI 100 for 18h prior to quantification of TNF-α secretion by ELISA. Results are expressed as the mean ± SD of three independent experiments. Stars indicate statistical significant differences when compared to *C. jejuni* as analyzed by ANOVA with Tukey post hoc test (A, B). C: Survival of *G. mellonella* larvae exposed to *C. concisus* isolates and *C jejuni* 11168H. *G. mellonella* larvae were injected with a bacterial suspension (~10^7 CFU) and their survival was monitored over time. Results represent the mean survival rate (n=30) pooled from two independent experiments. Survival rates in the *C. jejuni* group were compared to each of the *C. concisus* groups ($p<0.001$). The Mantel-Cox log-rank test was applied for statistical analysis. * $p<0.05$; ** $p<0.01$, *** $p<0.001$