

Cj1136 Is Required for Lipooligosaccharide Biosynthesis, Hyperinvasion, and Chick Colonization by *Campylobacter jejuni*

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***Campylobacter jejuni* is a major cause of bacterial food-borne enteritis worldwide, and invasion into intestinal epithelial cells is an important virulence mechanism. Recently we reported the identification of hyperinvasive *C. jejuni* strains and created a number of transposon mutants of one of these strains, some of which exhibited reduced invasion into INT-407 and Caco-2 cells. In one such mutant the transposon had inserted into a homologue of *cj1136*, which encodes a putative galactosyltransferase according to the annotation of the *C. jejuni* NCTC11168 genome. In the current study, we investigated the role of *cj1136* in *C. jejuni* virulence, lipooligosaccharide (LOS) biosynthesis, and host colonization by targeted mutagenesis and complementation of the mutation. The *cj1136* mutant showed a significant reduction in invasion into human intestinal epithelial cells compared to the wild-type strain 01/51. Invasion levels were partially restored on complementing the mutation. The inactivation of *cj1136* resulted in the production of truncated LOS, while biosynthesis of a full-length LOS molecule was restored in the complemented strain. The *cj1136* mutant showed an increase in sensitivity to the bile salts sodium taurocholate and sodium deoxycholate and significantly increased sensitivity to polymyxin B compared to the parental strain. Importantly, the ability of the mutant to colonize 1-day-old chicks was also significantly impaired. This study confirms that a putative galactosyltransferase encoded by *cj1136* is involved in LOS biosynthesis and is important for *C. jejuni* virulence, as disruption of this gene and the resultant truncation of LOS affect both colonization *in vivo* and invasiveness *in vitro*.**

C*ampylobacter jejuni* is a major cause of bacterial food-borne diarrhea worldwide. *Campylobacter* infections vary from asymptomatic to severe enteritis characterized by inflammatory watery diarrhea. Although *C. jejuni* enteritis is usually self-limiting, postinfection sequelae, including neuropathies such as Guillain-Barré syndrome and Miller-Fisher syndrome, can be debilitating (4).

Despite the high prevalence, clinical consequences, and economic importance of campylobacteriosis, the mechanisms of *C. jejuni* pathogenesis are not fully understood. *Campylobacter* invasion into the epithelial mucosa appears to be an essential part of the process leading to enteritis (28). The analysis of intestinal biopsy samples from patients infected with *C. jejuni* (50), as well as experiments with infected primates (44, 45) and other experimentally infected model animals (3, 35, 56), together with *in vitro* experiments using human epithelial cells (7, 28, 29, 36), has clearly demonstrated that *C. jejuni* can adhere to and invade the cells of the intestinal tract. Invasion studies with intestinal epithelial cells revealed a statistically significant correlation between the ability of *C. jejuni* to invade cultured cells and the severity of clinical symptoms of infection (10).

Poultry is considered the major source of food-borne campylobacteriosis (5, 37). The mechanisms by which *C. jejuni* initiates colonization, either in humans or in the avian host, are poorly understood, but a putative correlation between the ability of *C. jejuni* to invade cultured Caco-2 cells and colonization of chicks was previously proposed (18). Such a correlation between invasion of cell cultures and colonization ability was also found in other investigations (57).

Like many Gram-negative pathogenic bacteria, *C. jejuni* has surface polysaccharides, including lipooligosaccharide (LOS).

LOS consists of a highly variable outer core of nonrepeating oligosaccharides anchored to lipid A (8, 15, 38). Previously, the genes involved in LOS biosynthesis have been shown to confer variable virulence-associated phenotypes to *C. jejuni*. Inactivation of *galE* (encoding a UDP-glucose 4-epimerase) or *waaF* (encoding heptosyltransferase II) results in organisms expressing deeply truncated LOS molecules and with a reduced potential to invade epithelial cells *in vitro* (13). A recent study showed that both the *C. jejuni* 81-176 *waaF* and *lgtF* mutants produced truncated LOS and exhibited impaired colonization in the guts of BALB/c ByJ mice (34). Furthermore, complete deletion of the LOS biosynthesis locus in *C. jejuni* NCTC11168 led to attenuated growth and loss of invasion and natural transformation efficiency of the mutant (31). In contrast, mutants with mutations in *cgtA*, which encodes an outer core *N*-acetylgalactosaminyltransferase in *C. jejuni* 81-176, exhibited increased bacterial attachment and invasion of human cells, while in the same study mutation of *neuCI*, leading to the absence of sialic acid in the outer core, had no effect on invasiveness (17).

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
<i>C. jejuni</i>		
01/51	Hyperinvasive wild-type strain 01/51	11
01/51 Δ <i>cj1136</i>	<i>cj1136</i> mutation generated in strain 01/51	This study
01/51 Δ <i>cj1136::cj1136</i>	<i>cj1136</i> mutation complemented by wild-type copy of the gene in strain 01/51	This study
81116 Δ(<i>flaA flaB</i>)	Double mutation in <i>flaA</i> and <i>flaB</i> genes in strain 81116	52
<i>E. coli</i> TOP10F'	F' <i>lacI^q</i> Tn10(Tet ^r) <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
Plasmids		
pGEM-T Easy	Cloning vector encoding resistance to ampicillin	Promega
pGEM:1136::kan	pGEM-T Easy vector carrying 1.3-kb fragment of <i>cj1136</i> interrupted by Kan ^r cassette	This study
pAJ20	2.3-kb DNA fragment flanking the intergenic region between <i>cj0652</i> and <i>cj0653c</i> with additional BamHI restriction site in the intergenic region, cloned into pGEM-T Easy vector	This study
pAJ22	Chloramphenicol resistance cassette cloned into pAJ20	This study
pAJ22-1136	Wild-type copy of <i>cj1136</i> with its own promoter cloned into pAJ22 vector	This study
pMA24	Source of kanamycin resistance cassette	1
pUOA18	Source of chloramphenicol resistance cassette	51

To better understand the molecular basis of invasiveness in *C. jejuni*, we previously identified hyperinvasive strains (11) and generated a random transposon insertion library in the hyperinvasive strain 01/51. One of the mutants identified in a screen for reduced invasion of human intestinal epithelial cells harbored a transposon insertion into a gene homologous to *cj1136* of *C. jejuni* NCTC11168 (19). *cj1136* is part of the LOS biosynthesis locus (*cj1132c* to *cj1152c*) in *C. jejuni* NCTC11168 and shares homology with the genes for many bacterial β-1,3-galactosyltransferases (GalTs), but the function of the encoded protein and its importance in *C. jejuni* pathogenesis, host colonization, and LOS biosynthesis is unknown. In the current study, a *cj1136* homologue was characterized by mutagenesis, complementation, and phenotypic analysis to determine the involvement of this gene in LOS biosynthesis, virulence, and chicken colonization in *C. jejuni* 01/51. This *cj1136* homologue in 01/51 is 100% identical at the nucleotide level to *cj1136* of NCTC11168 and will therefore be referred to as *cj1136* for ease of description throughout this paper.

MATERIALS AND METHODS

Bacterial culture. *C. jejuni* strains (Table 1) were routinely grown on Columbia agar supplemented with sheep blood (5%, vol/vol) or Mueller-Hinton (MH) agar for 48 h at 37°C in an anaerobic work station (Don Whitley Scientific, United Kingdom) supplied with 10% CO₂, 5% O₂, and 85% (vol/vol) N₂ or in a gas jar where a CampyGen pack (Oxoid, United Kingdom) sachet was added to generate microaerobic conditions. *Escherichia coli* (Table 1) was grown on Luria-Bertani agar at 37°C in an aerobic atmosphere. Culture media were also supplemented with antibiotics, including ampicillin (100 μg ml⁻¹), kanamycin (50 μg ml⁻¹), and chloramphenicol (20 μg ml⁻¹), where appropriate.

Generation of *C. jejuni* 01/51 Δ*cj1136*. Briefly, the *cj1136* open reading frame, including 66 bp of upstream sequence and 90 bp of downstream sequence, was amplified by PCR from *C. jejuni* 01/51 using primers 1136-F and 1136-R (Table 2) and cloned into pGEM-T Easy (Promega). A BamHI restriction site was introduced into the cloned *cj1136* by inverse PCR (IPCR) (55) using primers 1136-INF and 1136-INR. IPCR generated a 4.3-kb PCR product flanked by BamHI restriction sites and containing a 57-bp deletion in *cj1136*. The Kan^r cassette was amplified from pMA24 (1) using Kan-F and Kan-R primers, BamHI digested, and ligated to BamHI-digested *cj1136* IPCR product. *E. coli* TOP10F' cells were transformed and transformants selected on plates containing kanamycin and ampicillin.

After screening, one clone containing the Kan^r cassette inserted in the same orientation as *cj1136* (named pGEM:1136::kan) was used to mutate *C. jejuni* 01/51 by electroporation and allelic exchange as described elsewhere (53). The mutation was confirmed by PCR analysis and nucleotide sequencing (data not shown).

Complementation of *C. jejuni* 01/51 Δ*cj1136*. A wild-type copy of *cj1136* (under the control of its own promoter) was inserted in the 121-bp intergenic region between *cj0652* and *cj0653c*. These are present in a tail-to-tail orientation in the chromosomes of *C. jejuni* NCTC11168 and 01/51. Briefly, a 2.3-kb fragment encompassing this region was amplified from *C. jejuni* 01/51 using 652-F and 653-F primers (Table 2) and cloned into pGEM-T Easy (Promega). A BamHI site was introduced in the intergenic region by IPCR using primers IN652-R and IN653-R and the resulting plasmid, pAJ20, confirmed by nucleotide sequencing. A chloramphenicol resistance (Cm^r) gene, amplified from pUOA18 (51) using primers CML-F and CML-R (which harbor BamHI and BglII restriction sites, respectively), was digested using BamHI and BglII and ligated to BamHI-digested pAJ20 to generate pAJ22 (Table 1). *cj1136* along with its presumptive promoter was amplified using primers C1136-F and C1136-R, digested with BamHI, and ligated into BamHI-digested pAJ22 to generate pAJ22-1136 (Table 1). This was electroporated into *C. jejuni*

TABLE 2 Primers used in this study

Primer	Nucleotide sequence (5' → 3') ^a
1136-F	GCTCGAAATCAATCATCAAATTT
1136-R	CAACTCTTTGGGAAGAAATTCAA
1136-INF	caaggatccGCACTATCACTCCAAGAGACTATG
1136-INR	caaggatccCCATCATCATAAATACAAGCATG
Kan-F	ggaggatccGATAAACCAGCGAACC
Kan-R	gcaggatccGACATCTAAATCTAGGTACTAAAACAATTC
C1136-F	caaggatccCGCAAAGTAATTTTACAAGGTTCA
C1136-R	caaggatccCAACTCTTTGGGAAGAAATTCAA
652-F	GAACCTACGATAGATATAGAGC
653-F	TTATGATGAAATCATCATGGAGC
IN652-R	caaggatccCAAGATTTTTAAACCAATTGC
IN653-R	caaggatccAATTATAAGTTAATACAAGG
CML-F	caaggatccGTCGGTATCGTATGGAGCG
CML-R	cacagatccCTAAAGGGTTTTATCAGTGCG

^a BamHI (ggatcc) or BglII (agatct) restriction sites and additional nucleotides at 5' ends of primers, where needed, are shown in lowercase.

01/51 $\Delta cj1136$ and the insertion of *cj1136* into the intergenic region between *cj0652* and *cj0653c* confirmed by PCR and nucleotide sequencing.

In vitro invasion and adhesion assay. The adhesion and invasion potentials of all strains were determined using *in vitro* invasion and adhesion assays as described earlier (19). Briefly, Caco-2 cell monolayers were grown in two 24-well cell culture plates. The Caco-2 cell monolayer was covered with bacterial cells at a multiplicity of infection of 100 in complete cell culture medium consisting of Eagle's minimal essential medium with glutamine supplemented with 10% fetal calf serum and 1% nonessential amino acids and incubated at 37°C for 3 h in 5% (vol/vol) CO₂. The monolayer was then washed 3 times with sterile phosphate-buffered saline (PBS), monolayers were lysed from one of the plates by adding 1% (vol/vol) Triton X-100, and viable bacterial cell counts were made to determine the adherent and invaded bacterial cell numbers. The monolayers of the other plate were covered with 1 ml of complete cell culture medium supplemented with 250 µg gentamicin ml⁻¹ in each well and incubated for a further 2 h. Following incubation, the monolayers were washed three times with PBS as before and lysed with 1 ml of 1% (vol/vol) Triton X-100 in PBS, and the total number of viable bacteria per well was determined.

Assays were performed in triplicate. The number of adherent bacteria was calculated by subtracting the number of internalized bacteria from the total number counted. Invasion efficiency was expressed as the percentage of the inoculum that survived gentamicin treatment.

Extraction of C. jejuni LOS. LOS was extracted using a modification of the phenol-water extraction procedure (32, 40). Briefly, confluent bacterial growth from agar plates was washed three times in PBS (Sigma, United Kingdom), and cells were resuspended in sterile distilled water to an optical density at 600 nm (OD₆₀₀) of 1.0. One milliliter of bacterial suspension was added to an equivalent volume of phenol (preheated to 65°C) (Sigma, United Kingdom) and mixed vigorously. Samples were incubated at 65°C for 10 min, mixed at regular intervals, and, after cooling on ice, centrifuged at 12,000 × g for 3 min. The upper aqueous layer was removed and LOS precipitated by the addition of 0.1 volume of 20% sodium acetate and 5 volumes of 100% ethanol at 4°C overnight. Precipitated LOS was harvested by centrifugation (5,000 × g) for 10 min, and the pellet washed with ice-cold 70% ethanol, dried, and resuspended in 500 µl water.

Analysis of C. jejuni LOS by SDS-PAGE. LOS extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were mixed with an equivalent volume of 2× sample loading buffer (100 mM Tris-HCl [pH 8.0], 2% β-mercaptoethanol, 4% SDS, 0.02% bromophenol blue, 20% glycerol) and boiled for 10 min before being loaded onto an SDS-polyacrylamide gel consisting of a stacking gel of 4% (vol/vol) acrylamide and a separating gel of 15% (vol/vol) acrylamide. The gel was run in SDS-Tris-glycine buffer (0.025 M Tris base, 0.192 M glycine, and 0.1% SDS, pH 8.3) at a constant 200 V per gel (Bio-Rad Protean II system). LOS was detected using a previously described silver staining method (48).

MS analysis of C. jejuni LOS. The intact LOS samples were analyzed using electrophoresis-assisted open-tubular liquid chromatography-mass spectrometry (EA-OTLC-MS) as described previously (9). Briefly, 1.0 µl of the LOS sample was injected into a capillary column, and this was followed by washing with 1.0 µl of 100% methanol. A small plug (60 nl) of 1 M ammonium acetate in deionized water was injected to elute the adsorbed intact LOS from the capillary surface. The separation was performed using 30 mM morpholine in deionized water, pH 9.0. A separation voltage of 30 kV, together with a pressure of 50,000 Pa, was applied for the EA-OTLC-MS analysis. The electrospray ionization (ESI) voltage applied on the sprayer was set at -5.2 kV. Data acquisition was performed in the range of *m/z* 1000 to 1600 at a scan rate of 2 s/spectrum. In the MS² (enhanced product ion scan [EPI]) experiments, the scan speed was set to 4,000 Da/s, with Q₀ trapping.

Resistance of C. jejuni to polymyxin B and bile salt detergents. Resistance to different concentrations of polymyxin B (range, 0.5 to 10 µg ml⁻¹) was determined by inoculating 10 µl of bacterial suspensions (at an

OD₅₅₀ of 0.1) onto MH agar supplemented with a specific concentration of polymyxin B. Growth was observed at 24, 48, and 72 h after incubation. All tests were carried out in triplicate. Resistance to different concentrations (range, 0 to 30 mg ml⁻¹) of sodium deoxycholate and sodium taurocholate was determined following the protocol described elsewhere (43).

Sensitivity to Triton X-100. To determine the sensitivity of *C. jejuni* to Triton X-100 (1%, vol/vol), overnight bacterial growth was harvested from blood agar plates and resuspended in 1 ml of distilled water to OD₅₅₀ of 0.1. After a zero-hour sample was taken, Triton X-100 was added and the bacterial cell suspension incubated at room temperature for 1 h. Viable cell counts were determined by serial dilution and plating. Assays were carried out in triplicate.

Motility assays. Motility assays were undertaken using Mueller-Hinton broth supplemented with 0.4% agar as described earlier (19).

Survival under environmental stress conditions. Overnight bacterial growth was harvested, and a bacterial cell suspension (OD₅₅₀ = 0.1) was prepared. For aeration stress, survival of strains was tested in Mueller-Hinton broth in a shaking incubator over 5 h. For survival in complete cell culture medium, survival was tested in medium in a static 5% CO₂ incubator over 5 h. Survival was determined by viable cell counts.

Chick colonization. Animal experiments were performed by licensed personnel in accordance with Home Office regulations and met with local ethics committee approval. Chick colonization was determined using a quantitative oral gavage model as previously described (54). Briefly, 4 groups of 10 specific-pathogen-free chicks, hatched from eggs obtained from Charles River SPAFAS Inc., Hanover, Germany, were housed in negative-pressure isolators and given food and water *ad libitum*. At 1 day old, birds were dosed by oral gavage with *C. jejuni* wild-type 01/51 or *C. jejuni* 01/51 $\Delta cj1136$ at $3.4 \pm 0.9 \times 10^4$ and $3.4 \pm 0.9 \times 10^6$ CFU in 100 µl of 0.1 M PBS, pH 7.2. Doses were prepared by harvesting bacteria grown overnight on blood agar plates into sterile PBS, and the actual doses were determined retrospectively by viable count. At 5 days postchallenge, the chicks were sacrificed by cervical dislocation and the colonization levels were determined by plating out dilutions of cecal contents onto blood agar plates (supplemented with kanamycin where appropriate). Individual chick colonization levels were determined as CFU per gram of cecal contents. The minimal level of detection was 100 CFU g⁻¹ cecal contents. Statistical analysis of the data was carried out with Minitab software using the nonparametric Mann-Whitney (two-tailed) test to assess significance. *P* values of <0.05 were judged to be statistically significant.

Statistical analysis. Unless otherwise stated, a paired Student *t* test was performed for statistical analysis. A *P* value of <0.05 indicated statistical significance.

RESULTS

Mutation and complementation of *cj1136* confirm a role in host cell invasion. *cj1136* was initially identified as being required for optimal invasion in a screen of *C. jejuni* transposon mutants (19). To confirm this phenotype, we created a new targeted *cj1136* mutant by insertional inactivation of the gene in strain 01/51. Phenotypic analysis confirmed that the mutant strain had a significantly reduced (*P* < 0.001) ability to invade Caco-2 cells compared to the wild type (Fig. 1B). Importantly, 01/51 $\Delta cj1136$ did not show a change in adhesion to Caco-2 cells compared to the wild type, confirming that the invasion-impaired phenotype was not a consequence of a reduced ability to adhere (Fig. 1A). Similarly, the *cj1136* mutant did not show a growth defect in MH broth or cell culture medium or exhibit reduced motility, increased sensitivity in Triton X-100, or reduced viability under the atmospheric conditions encountered during the course of the invasion assay compared to the wild type (data not shown). To rule out further polar effects and the possibility of secondary mutations, a wild-type copy of *cj1136* was introduced into the chromosome of 01/51

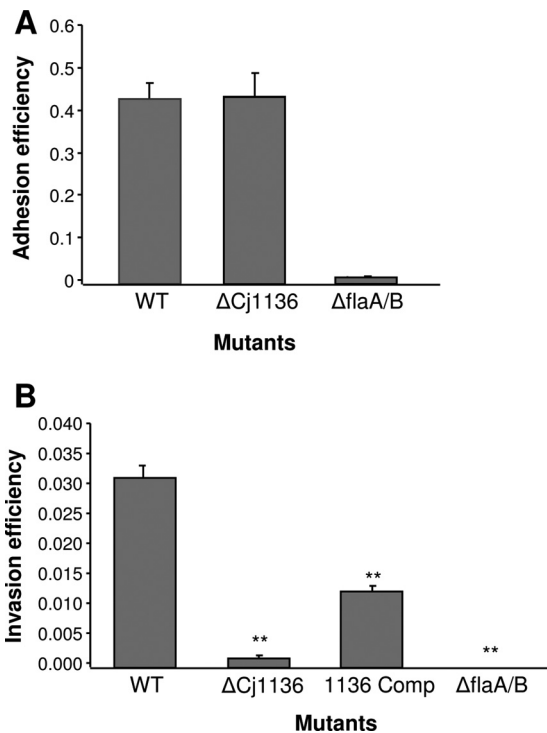


FIG 1 Adhesion and invasion assays using Caco-2 cells confirm the role of *cj1136* in host epithelial cell invasion but not adhesion. The adhesion phenotypes of *C. jejuni* wild-type (WT) 01/51 and *C. jejuni* 01/51 Δ *cj1136* (Δ Cj1136) (A) and the invasion phenotypes of *C. jejuni* WT 01/51, *C. jejuni* 01/51 Δ *cj1136*, and *C. jejuni* 01/51 Δ *cj1136::cj1136* (1136 Comp) (B) were determined using Caco-2 cells. A double mutant of *C. jejuni* 81116 with mutations in *flaA* and *flaB* (Δ flaA/B) was used as a negative control in both assays. A paired Student *t* test was performed to compare the invasion levels of (i) the mutant (Δ Cj1136) and negative control (Δ flaA/B) with that of the WT strain and (ii) the complemented mutant (1136 Comp) with that of the mutant (Δ Cj1136). **, $P < 0.001$. As there was no change in adhesion between the wild type and mutant, the complemented mutant was not tested in this assay.

Δ *cj1136*. Introduction of *cj1136* at an ectopic site restored, albeit not completely, invasion levels (Fig. 1B). Taken together, these results confirm the role of Cj1136 in invasion.

LOS analysis. Having confirmed the effect on host cell invasion, we purified the LOS produced by the wild-type, Δ *cj1136*, and Δ *cj1136::cj1136* strains and analyzed them by SDS-PAGE and silver staining as well as mass spectrometry.

(i) **Silver staining.** Inactivation of *cj1136* resulted in the generation of a truncated LOS molecule exhibiting an increased electrophoretic mobility compared to that of the LOS purified from the wild type in a silver-stained SDS-polyacrylamide gel (Fig. 2). In contrast, the LOS purified from the complemented strain was indistinguishable from that from the wild type using this analysis (Fig. 2). This confirms the putative role of Cj1136 in the biosynthesis of LOS.

(ii) **MS analysis of *C. jejuni* LOS.** To further characterize the nature of the difference in the LOSs produced by the wild-type strain and the *cj1136* mutant, LOS samples from *C. jejuni* wild-type 01/51, Δ *cj1136*, and Δ *cj1136::cj1136* strains were analyzed by using EA-OTLC-MS. The LOS sample purified from the wild-type strain showed related triply and quadruply deprotonated ions at *m/z* 1478 and 1108, respectively, which correspond to a species with a mass of ~4,436.5 Da, whereas LOS from the Δ *cj1136* mu-

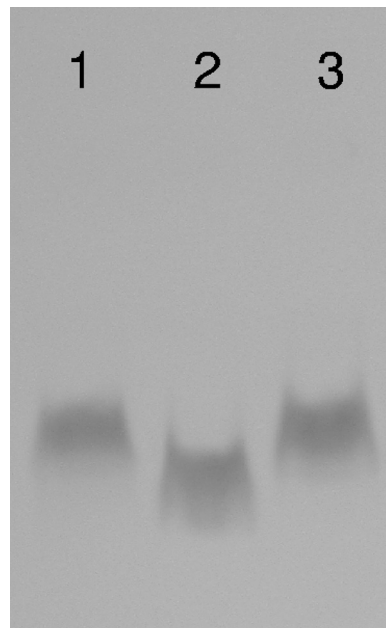


FIG 2 Mutation of *C. jejuni* 01/51 *cj1136* leads to the production of truncated LOS. LOSs extracted from *C. jejuni* wild-type 01/51 (lane 1), *C. jejuni* 01/51 Δ *cj1136* (lane 2), and *C. jejuni* 01/51 Δ *cj1136::cj1136* (lane 3) were separated on a 15% SDS-polyacrylamide gel and silver stained. LOS from the *cj1136* mutant showed an increased electrophoretic mobility compared to that extracted from the wild-type 01/51 or the complemented mutant.

tant showed a major triply deprotonated ion peak at *m/z* 1151, which corresponds to a species with a mass of ~3,456 Da. The MS data showed that the *cj1136* mutation resulted in the generation of truncated LOS which is ~980.5 Da smaller in mass than the wild-type LOS (Fig. 3) and confirmed the role of Cj1136 in LOS biosynthesis.

To further characterize the composition of LOS, tandem MS/MS was performed on the intact LOS from *C. jejuni* wild-type 01/51 (*m/z* 1108) and 01/51 Δ *cj1136* (*m/z* 1151). The LOS samples from both the wild type and the *cj1136* mutant showed a major peak at *m/z* 1062, the mass corresponding to the lipid A component containing a diamino-dideoxyhexose-amino-deoxyhexose (160.2 + 161.2 Da), two palmitate chains (2 × 238.4 Da), four 3-hydroxymyristate chains (4 × 226.4 Da), two phosphoethanolamine (PEtn) residues (2 × 123.05 Da), two phosphate (P) groups (2 × 79.98 Da), and a water molecule (18.0 Da), summing up to a mass of ~2,127.8 Da. The remaining mass of the core oligosaccharide in wild-type LOS is consistent with glycoforms containing two 2-keto-3-deoxyoctulosonic acid (KDO) residues (2 × 220.2 Da), one glycine (57.05 Da), one PEtn (123.05 Da), two heptose (Hep) residues (2 × 192.17 Da), five hexose (Hex) residues (5 × 162.14 Da), one *N*-acetylhexosamine (HexNAc) residue (203.19 Da), and one *N*-acetylneuraminic acid (Neu5Ac) residue (291.26 Da), summing up to a mass of ~2,309.6 Da. Thus, the mass of LOS from wild-type strain 01/51 corresponds to the composition Neu5Ac₁-HexNAc₁-Hex₅-Gly₁-Hep₂-PEtn₁-KDO₂-lipid A. The loss of a mass of ~980.5 Da in LOS generated by the *cj1136* mutant of strain 01/51 corresponds to three Hex residues (3 × 162.14 Da), one HexNAc residue (203.19 Da), and one Neu5Ac residue (291.26 Da). The total mass of truncated LOS produced in *C. jejuni* 01/51 Δ *cj1136* corresponds to the composition Hex₂-

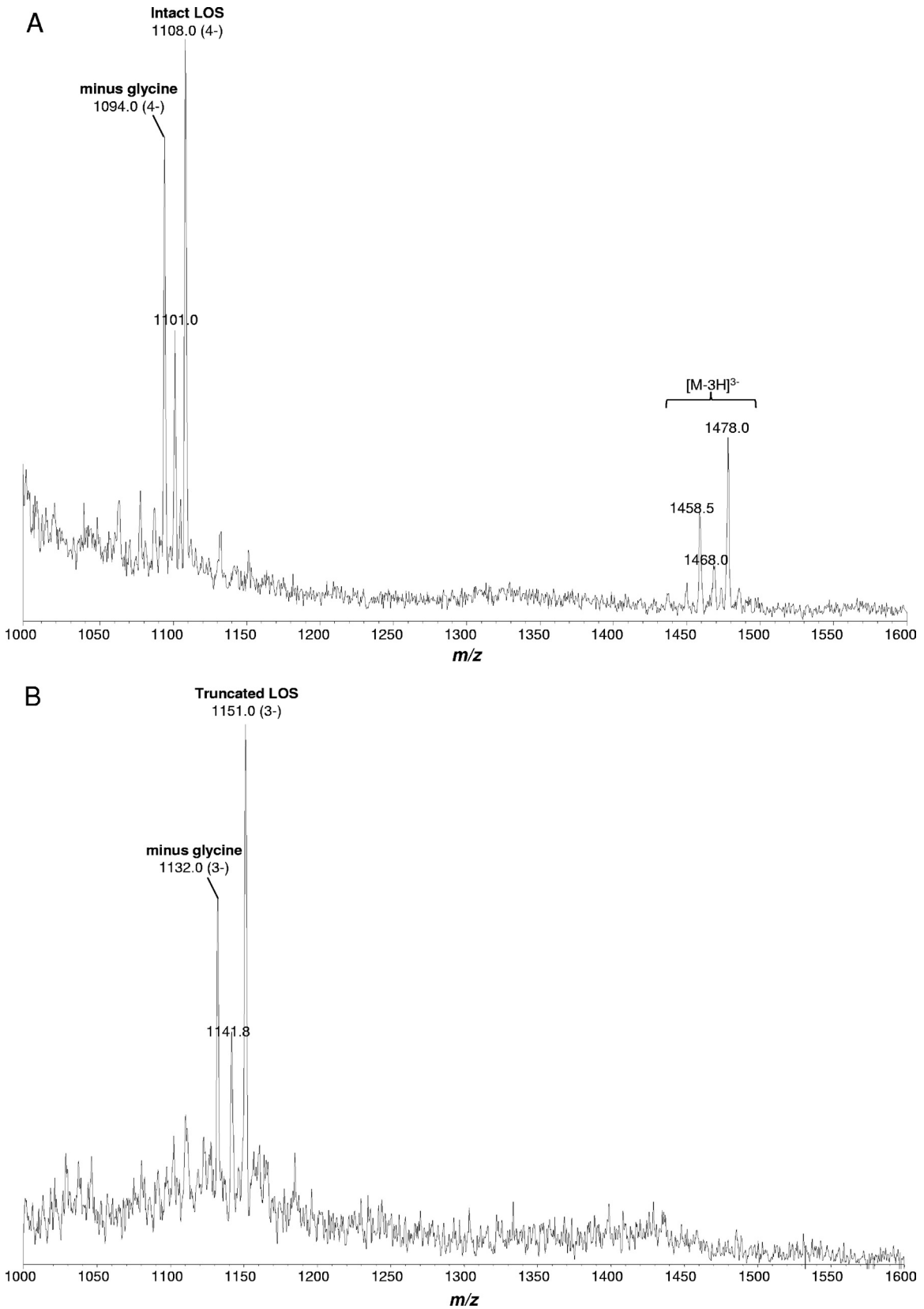


FIG 3 Electrophoresis-assisted open-tubular liquid chromatography-mass spectrometry analysis of intact LOSs from *C. jejuni* wild-type 01/51 (A) and *C. jejuni* 01/51 $\Delta cj1136$ (B). The MS analysis of LOS samples purified from the *C. jejuni* 01/51 wild-type strain showed major peaks at m/z 1108 and 1478 of quadruply and triply charged ions, respectively, whereas triply charged species were observed at m/z 1151 in LOS isolated from the *cj1136* mutant.

Gly₁-Hep₂-PEtn₁-KDO₂-lipid A. The LOS sample purified from the complemented mutant *C. jejuni* 01/51 Δ *cj1136::cj1136* showed the same molecular mass as that isolated from the wild-type 01/51 strain (not shown). The MS/MS data also showed the loss of monosaccharide hexose (*m/z* 219) and monosialic acid (Neu5Ac) (*m/z* 290) in the mutant (Fig. 4), which indicates the LOS truncation in the outer core.

Resistance to polymyxin B and bile salts. LOS forms a major part of the *C. jejuni* outer envelope and protects the bacteria from different antimicrobial peptides (34). We tested the sensitivities of our strains to polymyxin B to determine if production of truncated LOS by the mutant affected the sensitivity of the strain. The *cj1136* mutant showed a significant (6-fold) decrease in resistance to polymyxin B compared to the wild type (Table 3). The *cj1136* mutant showed growth in medium containing only 1.0 μ g polymyxin B ml⁻¹, whereas the wild-type strain 01/51 was able to grow at a concentration of 6 μ g polymyxin B ml⁻¹. Complementation of the *cj1136* mutation with a wild-type copy of the gene restored resistance to wild-type levels. Furthermore, the *cj1136* mutant also showed a decrease in resistance to the bile salts sodium deoxycholate and sodium taurocholate compared to strain 01/51 and the complemented mutant (Table 3).

***C. jejuni* 01/51 Δ *cj1136* is impaired in its ability to colonize the chick gut.** The *in vivo* significance of the truncated LOS brought about by the disruption of *cj1136* was tested using an *in vivo* model of chick colonization (54). Two groups of birds were dosed orally with the wild-type strain (one group with 10⁴ CFU and one group with 10⁶ CFU) and two with the *cj1136* mutant at doses similar to those for the wild type. The wild-type *C. jejuni* strain colonized 100% of the birds, and the median levels of colonization was 7.2 \times 10⁷ and 1.59 \times 10⁸ CFU g⁻¹ cecal content for the low and high doses, respectively. The colonization ability was impaired in the *cj1136* mutant, which showed a significantly (*P* < 0.001) lower level of chick colonization than the wild type (Fig. 5). Thirty percent of the birds (*n* = 3/10) challenged with the lower dose of the *cj1136* mutant were not detectably colonized. The median colonization levels reached in the birds challenged with either dose of the mutant were significantly below those observed in birds gavaged with similar doses of the wild-type strain (Fig. 5), suggesting that an intact LOS is required for optimal chick colonization by *C. jejuni*.

DISCUSSION

Previously, a study conducted in our laboratory using random transposon mutagenesis identified a number of genes involved in invasiveness in *C. jejuni* (19). A *C. jejuni* 01/51 homologue of NCTC11168 *cj1136* was found to be interrupted by transposon insertion in a mutant with a significantly reduced invasion phenotype in INT-407 and Caco-2 cells (19). Here we characterized the *cj1136* gene in detail by targeted mutagenesis, complementation, and phenotypic analysis.

In this study, mutation of *cj1136* resulted in reduced invasion in Caco-2 cells compared to the *C. jejuni* 01/51 wild-type strain (without a change in adhesion), confirming our original observations using the transposon mutant. Partial restoration of the invasion phenotype was observed in the complemented mutant; however, this was not to the level of the wild-type strain, which is most likely due to the introduction of the wild-type gene into a nonnative position in the genome. Even though some promoter sequence was included upstream of the *cj1136* gene in the comple-

mented mutant, the ectopic copy of *cj1136* may no longer be regulated or expressed in the exact same manner as the wild-type gene. This may not have led to a detectable difference in phenotype when the bacteria were grown on rich Mueller-Hinton agar (for LOS analysis and sensitivity testing) but may have done so in the invasion assays, when the medium utilized was Eagle's minimal essential medium.

Both adherence and invasion are multifactorial and motility-dependent processes (53), yet adherence is not always followed by invasion. No correlation between adhesion and invasion was established in our study, and elsewhere it has been reported that some adherent strains were also deficient in invasion into Caco-2 cells (18). LOS structures are generally important for invasion in many pathogenic bacteria (22, 30, 31, 41, 47). A previous study proposed a link between the presence of *cgtB*, encoding a putative galactosyltransferase in *C. jejuni*, and a higher level of invasion into human intestinal epithelial cells and chick colonization potential of the isolates (33). Inactivation of *galE* in *C. jejuni* resulted in the production of truncated LOS and reduced invasion into INT-407 cells (13), and mutation of *waaC* also reduced the invasion potential of *C. jejuni* 81-176 (24). In contrast, a site-specific insertional mutation of the *cgtA* gene in *C. jejuni* strain 81-176, which encodes an *N*-acetylgalactosaminyltransferase, led to a significant (more than 2-fold) increase in invasion of INT-407 cells compared to that of the wild type (17). These studies suggest that a minimal LOS core structure is required for optimal invasion and that mutations that cause truncation of this minimal structure (mutations in *waaC*, *waaF*, *galE*, and, as now shown, *cj1136*) lead to impaired invasion. Mutations in the outer core (e.g., *cgtA*) are more likely to have strain-specific effects, modulating invasion in some but not all strains.

Lipopolysaccharide (LPS) and LOS are important for host colonization in many Gram-negative pathogens. In *Salmonella* species, a reduction in the ability to colonize chickens correlated with changes in the LPS profile (6, 49). A recent study has shown the importance of LOS for host colonization in *C. jejuni* using a mouse competition model (34). We have studied colonization in the chicken, which is the natural host and main source of human *C. jejuni* infection. *C. jejuni* 01/51 Δ *cj1136* showed a significant reduction, indicating that *cj1136* and an intact LOS are crucial for chick colonization by this organism.

cj1136 is part of the LOS biosynthesis locus in *C. jejuni* NCTC11168 (39). Based on nucleotide sequence homology to bacterial galactosyltransferase genes, *cj1136* was predicted to transfer β -1,3-galactose to heptose II in LOS biosynthesis (15, 26). We determined the compositions of LOS samples purified from the *C. jejuni* 01/51 wild-type strain as well as from the *cj1136* mutant and the complemented mutant by mass spectrometry, and the mutant showed the loss of 1 Neu5Ac, 1 HexNAc, and 3 Hex residues from its LOS outer core. These data suggest that *Cj1136* is a hexose transferase; however, further structural and linkage analysis of LOS will help determine the exact hexose residue transferred and linkage formed. Nevertheless, these data provide the first experimental confirmation that *cj1136* is involved in LOS biosynthesis in *C. jejuni* and that inactivation of this gene results in a truncated LOS.

The structure of LOS varies between *C. jejuni* strains, and this variability can result from difference in gene content, phase variation due to homopolymeric tracts, amino acid substitution, or mutation in glycosyltransferase-encoding genes. *cj1136* is not

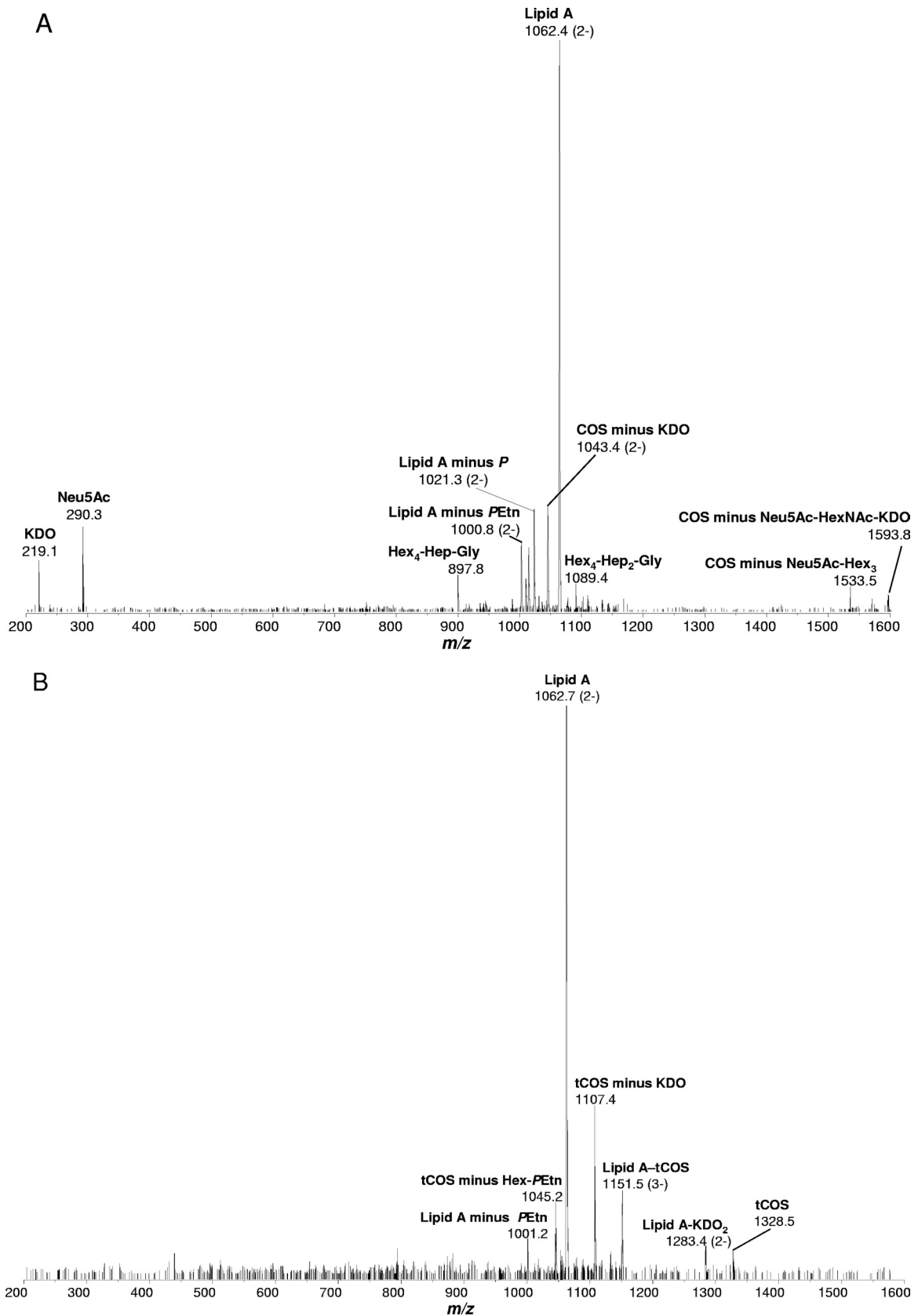


FIG 4 MS/MS spectra from the intact LOSs from *C. jejuni* wild-type 01/51 (A) and *C. jejuni* 01/51 $\Delta cj1136$ (B). The intact LOS of *C. jejuni* wild-type 01/51 ions m/z 1108 and truncated LOS of *cj1136* mutant ions m/z 1151 were further analyzed by MS/MS. The major cluster of molecular ions at m/z 1062.4 corresponds to lipid A in the LOSs from both wild-type 01/51 and its *cj1136* mutant. Neu5Ac, sialic acid; KDO, 2-keto 3-deoxy-octulosonic acid, P, phosphate; PEtn, phosphoethanolamine; COS, core oligosaccharide; tCOS, truncated core oligosaccharide; Hex, hexose; Hep, heptose; Gly, glycine. The composition of COS corresponds to Neu5Ac₁-HexNAc₁-Hex₅-Gly₁-Hep₂-PEtn₁-KDO₂, and that of tCOS corresponds to Hex₂-Gly₁-Hep₂-PEtn₁-KDO₂.

TABLE 3 Sensitivities of *C. jejuni* 01/51, 01/51 $\Delta cj1136$, and the complemented mutant (01/51 $\Delta cj1136::cj1136$) to polymyxin B and bile salts

Compound	Maximum concn ($\mu\text{g ml}^{-1}$) of compound at which growth was observed		
	Wild-type 01/51	01/51 $\Delta cj1136$	01/51 $\Delta cj1136::cj1136$
Polymyxin B	6.0	1.0	6.0
Sodium deoxycholate	28	20	28
Sodium taurocholate	30	22	30

phase variable (15). Interestingly, in this study we found that the LOS of *C. jejuni* 01/51 has a glycine modification. The glycine modifications of LOSs from other *C. jejuni* strains have previously been reported (9); however, the role of LOS modification with glycine in *C. jejuni* pathogenesis is as yet unknown.

LOS plays an important role in maintaining the integrity of the outer membrane. It also provides protection to the organism from antimicrobial peptides and bile salt detergents. Polymyxin B is an antimicrobial peptide that has binding affinity to negatively charged structures, including LPS/LOS. The *cj1136* mutant showed a 6-fold reduction in resistance to polymyxin B compared to wild-type strain 01/51. This finding is consistent with other previous studies that also reported a higher sensitivity to polymyxin B in mutants with truncated LOS (20, 31, 34), confirming that an intact LOS is important in the protection of the organism from antimicrobial peptides.

Bile salts, secreted in bile in the animal intestine, also have potential bactericidal activity. They can kill bacterial organisms by destroying the lipid bilayer outer membrane. The *cj1136* mutant showed greater sensitivity to the bile salt detergents sodium deoxycholate and sodium taurocholate, which is in line with other studies (23, 31, 46). The increased sensitivity observed in the *cj1136* mutant may be explained by a recent hypothesis that the negatively charged cell surface molecules normally buried by LOS are exposed to allow binding and thus disruption of the cell membrane by polymyxin B and bile salt detergents (2). However, the product of *cj1136* or LOS may also have secondary roles involved in efflux pumps and/or electrostatic interactions of bacterial surface molecules.

Mutants with mutations in putative glycosyltransferase genes *waaF*, *lgtF*, and *galT* in *C. jejuni* 81-176 have been reported to have reduced invasion potential compared to the wild-type strain (22, 24), and this reduction in invasiveness was found to be due to increased sensitivity to Triton X-100 used for lysis of the cultured cell monolayer. We tested the survival of the *cj1136* mutant in Triton X-100 and found the mutation did not alter sensitivity, confirming that the reduction in invasion levels exhibited by the mutant was not due to killing of the mutant by Triton X-100. Furthermore, the reduction in invasion exhibited by the mutant was not due to a change in growth, motility, or survivability under the environmental conditions encountered during the invasion assays.

A number of other genes as well as *cj1136* encode putative galactosyltransferases, including *galT* in *C. jejuni* 81-176 (14), and eight genes encode putative galactosyltransferases (*wlaH*, *wlaG*, *cj1136*, *cj1138*, *wlaN* [*cj1139c*], *cj1434c*, *cj1438c*, and *cj1440c*) in *C. jejuni* NCTC11168 (39). Sequencing of *C. jejuni* RM1221 resulted

in the annotation of two further putative galactosyltransferase genes (*cje1278* and *cje1280*) with no similarity to genes of NCTC11168 (12). Apart from *cj1136*, those genes encoding putative galactosyltransferases in *C. jejuni* 01/51 are not known, but if others do exist, they were unable to compensate for the *cj1136* mutation in this strain.

Capsular polysaccharide of *C. jejuni* has previously been shown to play a role in *C. jejuni* pathogenesis and host colonization (21) and is believed to be the major serodeterminant in the Penner or heat-stable (HS) serotyping scheme (27). Furthermore, in addition to LOS biosynthesis, a putative galactosyltransferase encoded by *cj1136* might also be involved in the biosynthesis of other carbohydrate structures, including capsular polysaccharides. Heptosyltransferase I encoded by *waaC* in *C. jejuni* 81-176 was found to be involved in biosynthesis of both the LOS and capsular carbohydrates (23), and an epimerase encoded by *gne* was found to be important for the three major carbohydrates' biosynthetic pathways (LOS, capsule, and N-linked protein glycosylation) (16). Similarly, a single phosphatase enzyme, GmhB, is involved in both LOS and capsule biosynthetic pathways (25). Interestingly, the serotype of the *C. jejuni* 01/51 wild-type strain is HS4 but the *cj1136* mutant's serotype is HS50, which raises the question as to whether the capsule structure is also altered in the *cj1136* mutant strain or whether the LOS is the major serodeterminant in strain 01/51. HS4 and HS50 are known to be cross-reacting serotypes, and it has been suggested previously that LOS does play a role in their serospecificity (42), so it is probable that a change in the LOS structure does bring about a change in reactivity with the serotyping antisera; nevertheless, the capsule region of *C. jejuni* 01/51 is currently being investigated. It is possible that *cj1136* may have a more direct role in virulence and host colonization. However, at

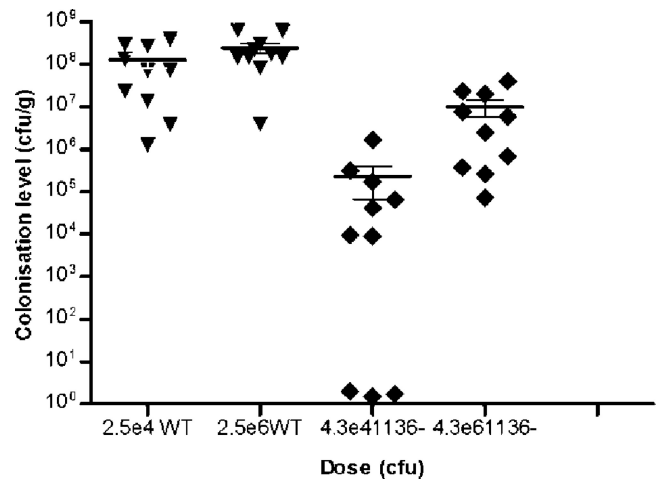


FIG 5 Mutation of *cj1136* results in a reduced ability to colonize chicks. The *C. jejuni* wild-type (WT) 01/51 and its *cj1136* mutant derivative (1136-) were tested in an *in vivo* model of chick colonization. A low dose and a high dose of each strain were administered to 10 birds in each group. The number of bacteria that had colonized the birds was determined in cecal contents at 5 days after infection. The *C. jejuni* 01/51 wild-type strain colonized to $\sim 10^8$ CFU g^{-1} irrespective of dose; however, both low and high doses of the mutant in *cj1136* showed significantly lower levels of chick colonization compared to cognate doses of the wild-type strain, confirming that *cj1136* plays a role in chick colonization by *C. jejuni*. A nonparametric Mann-Whitney (two-tailed) test was used to assess statistical significance of colonization levels of the mutant compared with the wild type at each dose ($P < 0.001$).

this stage it appears that the observed reduction in virulence and host colonization in the *cj1136* mutant of *C. jejuni* 01/51 is due to the production of truncated LOS.

This study, for the first time, confirmed the role of *cj1136* in LOS biosynthesis, the invasion of human intestinal epithelial cells *in vitro*, and the *in vivo* colonization of the chicken gut. It also emphasizes the importance of LOS as a fundamental structure of *C. jejuni* that affects many different phenotypes of this major human pathogen.

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REFERENCES

1. Abuoun M. 2007. The heterogeneity of the putative *Campylobacter* virulence factor: cytolethal distending toxin (CDT). Ph.D. thesis. University of Warwick, Warwick, United Kingdom.
2. Amini S, Goodarzi H, Tavazoie S. 2009. Genetic dissection of an exogenously induced biofilm in laboratory and clinical isolates of *E. coli*. PLoS Pathog. 5:e1000432. doi:10.1371/journal.ppat.1000432.
3. Babakhani FK, Joens LA. 1993. Primary swine intestinal cells as a model for studying *Campylobacter jejuni* invasiveness. Infect. Immun. 61:2723–2726.
4. Blaser MJ, Engberg J. 2008. Clinical aspects of *C. jejuni* and *C. coli* infections, p 99–121. In Nachamkin I, Szymanski CM, Blaser MJ (ed), *Campylobacter*. ASM Press, Washington, DC.
5. Butzler JP. 2004. *Campylobacter*, from obscurity to celebrity. Clin. Microbiol. Infect. 10:868–876.
6. Craven SE, et al. 1993. Characterization of *Salmonella californica* and *S. typhimurium* strains with reduced ability to colonize the intestinal tract of broiler chicks. Avian Dis. 37:339–348.
7. De Melo MA, Gabbiani G, Pechere JC. 1989. Cellular events and intracellular survival of *Campylobacter jejuni* during infection of HEp-2 cells. Infect. Immun. 57:2214–2222.
8. Dorrell N, et al. 2001. Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. Genome Res. 11:1706–1715.
9. Dzieciatkowska M, et al. 2007. Mass spectrometric analysis of intact lipooligosaccharide: direct evidence for O-acetylated sialic acids and discovery of O-linked glycine expressed by *Campylobacter jejuni*. Biochemistry 46:14704–14714.
10. Everest PH, et al. 1992. Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli*. J. Med. Microbiol. 37:319–325.
11. Fearnley C, et al. 2008. Identification of hyperinvasive *Campylobacter jejuni* strains isolated from poultry and human clinical sources. J. Med. Microbiol. 57:570–580.
12. Fouts D, et al. 2005. Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. PLoS Biol. 3:e15. doi:10.1371/journal.pbio.0030015.
13. Fry BN, et al. 2000. The *galE* gene of *Campylobacter jejuni* is involved in lipopolysaccharide synthesis and virulence. Infect. Immun. 68:2594–2601.
14. Gilbert M, et al. 2002. The genetic bases for the variation in the lipooligosaccharide of the mucosal pathogen, *Campylobacter jejuni*. Biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. J. Biol. Chem. 277:327–337.
15. Gilbert M, Parker CT, Moran AP. 2008. *Campylobacter jejuni* lipooligosaccharides: structure and biosynthesis, p 483–504. In Nachamkin I, Szymanski CM, Blaser MJ (ed), *Campylobacter*. ASM Press, Washington, DC.
16. Guerry P, Szymanski CM. 2008. *Campylobacter* sugars sticking out. Trends Microbiol. 16:428–435.
17. Guerry P, et al. 2002. Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness *in vitro*. Infect. Immun. 70:787–793.
18. Hanel I, Muller J, Muller W, Schulze F. 2004. Correlation between invasion of Caco-2 eukaryotic cells and colonization ability in the chick gut in *Campylobacter jejuni*. Vet. Microbiol. 101:75–82.
19. Javed MA, et al. 2010. Transposon mutagenesis in a hyper-invasive clinical isolate of *Campylobacter jejuni* reveals a number of genes with potential roles in invasion. Microbiology 156:1134–1143.
20. Jeon B, Muraoka W, Scupham A, Zhang Q. 2009. Roles of lipooligosaccharide and capsular polysaccharide in antimicrobial resistance and natural transformation of *Campylobacter jejuni*. J. Antimicrob. Chemother. 63:462–468.
21. Jones MA, et al. 2004. Adaptation of *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian gastrointestinal tract. Infect. Immun. 72:3769–3776.
22. Kanipes MI, Holder LC, Corcoran AT, Moran AP, Guerry P. 2004. A deep-rough mutant of *Campylobacter jejuni* 81-176 is noninvasive for intestinal epithelial cells. Infect. Immun. 72:2452–2455.
23. Kanipes MI, Papp-Szabo E, Guerry P, Monteiro MA. 2006. Mutation of *waaC*, encoding heptosyltransferase I in *Campylobacter jejuni* 81-176, affects the structure of both lipooligosaccharide and capsular carbohydrate. J. Bacteriol. 188:3273–3279.
24. Kanipes MI, et al. 2008. Genetic analysis of lipooligosaccharide core biosynthesis in *Campylobacter jejuni* 81-176. J. Bacteriol. 190:1568–1574.
25. Karlyshev AV, et al. 2005. Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. Mol. Microbiol. 55:90–103.
26. Karlyshev AV, Ketley JM, Wren BW. 2005. The *Campylobacter jejuni* glycome. FEMS Microbiol. Rev. 29:377–390.
27. Karlyshev AV, Linton D, Gregson NA, Lastovica AJ, Wren BW. 2000. Genetic and biochemical evidence of a *Campylobacter jejuni* capsular polysaccharide that accounts for Penner serotype specificity. Mol. Microbiol. 35:529–541.
28. Ketley JM. 1997. Pathogenesis of enteric infection by *Campylobacter*. Microbiology 143:5–12.
29. Konkel ME, Joens LA. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. Infect. Immun. 57:2984–2990.
30. Lambotin M, et al. 2005. Invasion of endothelial cells by *Neisseria meningitidis* requires cortactin recruitment by a phosphoinositide-3-kinase/Rac1 signalling pathway triggered by the lipo-oligosaccharide. J. Cell Sci. 118:3805–3816.
31. Marsden GL, Li J, Everest PH, Lawson AJ, Ketley JM. 2009. Creation of a large deletion mutant in *Campylobacter jejuni* reveals the lipooligosaccharide gene cluster is not required for viability. J. Bacteriol. 191:2392–2399.
32. McNally A, La Ragione RM, Best A, Manning G, Newell DG. 2007. An aflagellate mutant *Yersinia enterocolitica* biotype 1A strain displays altered invasion of epithelial cells, persistence in macrophages, and cytokine secretion profiles *in vitro*. Microbiology 153:1339–1349.
33. Muller J, Meyer B, Hanel I, Hotzel H. 2007. Comparison of lipooligosaccharide biosynthesis genes of *Campylobacter jejuni* strains with varying abilities to colonize the chicken gut and to invade Caco-2 cells. J. Med. Microbiol. 56:1589–1594.
34. Naito M, et al. 2010. Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. J. Bacteriol. 192:2182–2192.
35. Newell DG, Pearson A. 1984. The invasion of epithelial cell lines and the intestinal epithelium of infant mice by *Campylobacter jejuni/coli*. J. Diarrhoeal Dis. Res. 2:19–26.
36. Oelschlaeger TA, Guerry P, Kopecko DJ. 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc. Natl. Acad. Sci. U. S. A. 90:6884–6888.
37. Park SF. 2002. The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. Int. J. Food Microbiol. 74:177–188.
38. Parker CT, Gilbert M, Yuki N, Endtz HP, Mandrell RE. 2008. Characterization of lipooligosaccharide-biosynthetic loci of *Campylobacter jejuni* reveals new lipooligosaccharide classes: evidence of mosaic organizations. J. Bacteriol. 190:5681–5689.
39. Parkhill J, et al. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 403:665–668.
40. Prendergast MM, Kosunen TU, Moran AP. 2001. Development of an immunoassay for rapid detection of ganglioside GM(1) mimicry in *Campylobacter jejuni* strains. J. Clin. Microbiol. 39:1494–1500.
41. Preston A, Mandrell RE, Gibson BW, Apicella MA. 1996. The lipooli-

- gosaccharides of pathogenic gram-negative bacteria. *Crit. Rev. Microbiol.* 22:139–180.
42. Preston MA, Penner JL. 1989. Characterization of cross-reacting serotypes of *Campylobacter jejuni*. *Can. J. Microbiol.* 35:265–273.
 43. Raphael BH, et al. 2005. The *Campylobacter jejuni* response regulator, CbrR, modulates sodium deoxycholate resistance and chicken colonization. *J. Bacteriol.* 187:3662–3670.
 44. Russell RG, Blaser MJ, Sarmiento JI, Fox J. 1989. Experimental *Campylobacter jejuni* infection in *Macaca nemestrina*. *Infect. Immun.* 57:1438–1444.
 45. Russell RG, O'Donnoghue M, Blake DC, Jr, Zulty J, DeTolla LJ. 1993. Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant *Macaca mulatta*. *J. Infect. Dis.* 168:210–215.
 46. Steeghs L, et al. 2001. Outer membrane composition of a lipopolysaccharide-deficient *Neisseria meningitidis* mutant. *EMBO J.* 20:6937–6945.
 47. Swords WE, et al. 2000. Non-typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. *Mol. Microbiol.* 37:13–27.
 48. Tsai C-M, Frasch CE. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119:115–119.
 49. Turner AK, Lovell MA, Hulme SD, Zhang-Barber L, Barrow PA. 1998. Identification of *Salmonella* Typhimurium genes required for colonization of the chicken alimentary tract and for virulence in newly hatched chicks. *Infect. Immun.* 66:2099–2106.
 50. van Spreuwel JP, et al. 1985. *Campylobacter colitis*: histological immunohistochemical and ultrastructural findings. *Gut* 26:945–951.
 51. Wang Y, Taylor DE. 1990. Chloramphenicol resistance in *Campylobacter coli*: nucleotide sequence, expression, and cloning vector construction. *Gene* 94:23–28.
 52. Wassenaar TM, Bleumink-Pluym NM, Newell DG, Nuijten PJ, van der Zeijst BA. 1994. Differential flagellin expression in a *flaA flaB*⁺ mutant of *Campylobacter jejuni*. *Infect. Immun.* 62:3901–3906.
 53. Wassenaar TM, Bleumink-Pluym NM, van der Zeijst BA. 1991. Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. *EMBO J.* 10:2055–2061.
 54. Wassenaar TM, van der Zeijst BA, Ayling R, Newell DG. 1993. Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. *J. Gen. Microbiol.* 139:1171–1175.
 55. Wren BW, Henderson J, Ketley JM. 1994. A PCR-based strategy for the rapid construction of defined bacterial deletion mutants. *Biotechniques* 16:994–996.
 56. Yao R, Burr DH, Guerry P. 1997. CheY-mediated modulation of *Campylobacter jejuni* virulence. *Mol. Microbiol.* 23:1021–1031.
 57. Ziprin RL, et al. 2001. Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. *Avian Dis.* 45:549–557.