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Campylobacter jejuni Glycosylation Island Important in Cell Charge, Legionaminic Acid Biosynthesis, and Colonization of Chickens[▽]

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Previously, we identified five genes (Cj1321 to Cj1326, of which Cj1325 and Cj1326 are a single gene) in the O-linked flagellin glycosylation island that are highly prevalent in *Campylobacter jejuni* isolates from chickens. We report mutagenesis, functional, and structural data to confirm that this locus, and Cj1324 in particular, has a significant contributory role in the colonization of chickens by *C. jejuni*. A motile ΔCj1324 mutant with intact flagella was considerably less hydrophobic and less able to autoagglutinate and form biofilms than the parent strain, 11168H, suggesting that the surface charge of flagella of Cj1324-deficient strains was altered. The physical and functional attributes of the parent were restored upon complementation. Structural analysis of flagellin protein purified from the ΔCj1324 mutant revealed the absence of two legionaminic acid glycan modifications that were present in the parent strain, 11168H. These glycoform modifications were shown to be prevalent in chicken isolates and confirm that differences in the highly variable flagellin glycosylation locus can relate to the strain source. The discovery of molecular mechanisms influencing the persistence of *C. jejuni* in poultry aids the rational design of approaches to control this problematic pathogen in the food chain.

Campylobacter jejuni is the leading bacterial cause of human gastroenteritis worldwide (7). Infection can cause symptoms including abdominal pain and fever with watery to bloody diarrhea (54). Occasionally, postinfectious sequelae follow *C. jejuni* infection and include reactive arthritis and Guillain-Barré syndrome (8). Recently, *C. jejuni* has been associated with immunoproliferative small intestine disease, which is a rare type of mucosa-associated lymphoid tissue lymphoma (31). The main source of transmission through the food chain is the consumption and handling of contaminated poultry, but the underlying reasons why chickens are particularly susceptible to colonization by *C. jejuni* are unknown (15). *C. jejuni* has also been recovered from nonavian livestock, unpasteurized milk, and contaminated water (7). The socioeconomic burden of this pathogen means that it is imperative that ways of reducing the levels of *C. jejuni* in the food chain, particularly poultry, are investigated.

The glycosylation of flagellin in a number of gram-negative pathogenic bacteria, including *Pseudomonas aeruginosa*, *Helicobacter pylori*, and *Aeromonas* spp., is increasingly recognized as playing significant roles (2, 24, 32, 43, 49). Glycosylation modifications have been shown to influence the cell's immunogenicity, interaction with eukaryotic cells, and host cell specificity. Aeromonads are waterborne bacteria that can cause disease in fish, reptiles, and amphibians. Mesophilic aeromonads are important human pathogens causing gastrointestinal infections and, in severe cases, wound disease and septicemia in healthy and immunocompromised patients (63). Flagella of the mesophilic aeromonad *Aeromonas caviae* have been shown to be glycosylated (43) with a derivative of pseudaminic acid (50). In the plant pathogen *Pseudomonas syringae* pv. glycinea, the mutation of three genes located in a flagellin glycosylation island results in alterations to host specificity (61). Mutants of *P. syringae* pv. glycinea fail to cause symptoms in the normal host, soybean plants, but can grow on nonhost tobacco leaves, causing symptom-like changes on leaves. Takeuchi et al. proposed that the posttranslational modification of flagellin may be an adaptation of the bacterium to avoid recognition by host defenses (61). In *P. aeruginosa* strain PAK, a flagellin glycosylation island comprising 14 genes was discovered and shown to cause glycosylation exclusively for *P. aeruginosa* isolates expressing a-type flagellin (2). Further studies have shown that there appears to be variation in the glycosylation islands of strains containing the a-type flagellin (4). A glycosylation island comprising four genes in the type b flagellin strain *P. aeruginosa* PAO has been found. When a mutant unable to glycosylate flagellin was tested in a murine model of burn wound infection, it exhibited a reduction in virulence compared to that of the wild type (3). Thus, it appears that in *P. aeruginosa* different glycoforms on the flagellin

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are required for the colonization of different hosts or environments and that these glycoforms may provide the bacterium with a specific survival advantage.

We recently examined 111 strains of *C. jejuni*, including human, chicken, bovine, ovine, and environmental isolates, using comparative phylogenomics (whole-genome comparisons of microbes using DNA microarrays combined with Bayesian-based phylogenies) (10). Isolates fell into two distinct clades, which based on the origins of the isolates were defined as livestock-associated and non-livestock-associated clades. Over 40 genes were identified as being significantly prevalent in either of the clades. Among these was a set of six genes, Cj1321, Cj1322, Cj1323, Cj1324, Cj1325, and Cj1326 (as identified in the initial annotation by Parkhill et al. for the original sequenced *C. jejuni* strain, NCTC11168 [42]), that lie within a region of the genome encoding the flagellin O-linked glycosylation system. Thus, although genes Cj1321 to Cj1326 are located within a region of the genome which has variability, they are conserved among some *C. jejuni* strains that are often associated with livestock. Microarray data have shown that the six genes are all transcribed in the same orientation, but it is unknown if they are an operon (N. Dorrell and B. W. Wren, unpublished data). NCTC11168 has since been reannotated, and as a result, Cj1325 and Cj1326 are now considered to be one gene, hereinafter referred to as Cj1325/6 (22). Previous BLAST analyses have shown that the Cj1321 protein has amino acid similarity to many bacterial acetyl transferases, both Cj1322 and Cj1323 proteins are similar to hydroxyacyl dehydrogenases, and the product of Cj1324 is similar to WbpG, a protein involved in lipopolysaccharide synthesis in many bacteria.

In *C. jejuni* NCTC11168 (the original sequenced strain, found in the livestock clade), the O-linked flagellar glycosylation system is thought to consist of a cluster of approximately 50 genes (Cj1293 to Cj1342) adjacent to *flaA* and *flaB* which encode the structural flagellin proteins (42). The full glycan structure(s) in NCTC11168 (and most other strains associated with livestock) is unknown, but given the considerably larger size of the O-linked glycosylation loci in the livestock-associated strains than in the non-livestock-associated strains, it is likely that the livestock-associated strains may have additional modifications to the pseudaminic acid basic structure, as well as other unique glycan moieties, compared to those of the non-livestock-associated strains. The flagellin O-linked glycosylation locus in *C. jejuni* 81-176 (a frequently studied human strain found in the non-livestock-associated clade) is far simpler than that in *C. jejuni* NCTC11168, comprising just 26 genes (21). Two modifications predominantly decorate FlaA and FlaB of strain 81-176, the nine-carbon sugar pseudaminic acid (5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-α-L-*manno*-nonulosonic acid [Pse5Ac7Ac]) and an acetamidino form of pseudaminic acid, 5-acetamido-7-acetamidino-3,5,7,9-tetradeoxy-L-*glycero*-α-L-*manno*-nonulosonic acid (Pse5Ac7Am). Derivatives of Pse5Ac7Am also decorate the flagellin of 81-176 in minor quantities (34, 37, 62). Genetic analysis of 81-176 showed that *pse* genes are involved in the biosynthesis of pseudaminic acid and its derivatives (21, 37, 62). More recently, the full biosynthetic pathway for pseudaminic acid was determined; in a six-step reaction, UDP-*N*-acetylglucosamine (UDP-GlcNAc) is converted to pseudaminic acid through the actions of PseB/Cj1293, PseC/Cj1294, PseH/Cj1313, PseG/Cj1312, PseI/Cj1317, and PseF/Cj1311 proteins (The Cj designations refer to predicted coding sequences in *C. jejuni* NCTC11168) (11, 19, 21, 35, 52, 62).

The most detailed analysis of the flagellin O-linked glycosylation locus has been undertaken with *C. jejuni* strain 81-176 and the related species *Campylobacter coli* (strain VC167) (34). Structural studies of the flagellum modifications of *C. coli* VC167 revealed that in addition to Pse5Ac7Ac, acetamidino and *N*-methylacetimidoyl derivatives of legionaminic acid [5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-D-*glycero*-D-*galacto*-nonulosonic acid (Leg5Am7Ac) and 5-*E*/*Z*-*N*-(*N*-methlyacetimidoyl)-7-acetamidino-3,5,7,9-tetradeoxy-D-*galacto*-nonulosonic acid (Leg5AmNMe7Ac), respectively] decorate the *C. coli* flagellin, the first demonstration of a legionaminic acid derivative modification of bacterial flagellin (36). Biosynthesis of these legionaminic acid derivatives involves a distinct pathway encoded by the posttranslational modification (*ptm*) genes (34, 36). Although the precise pathway for the production of legionaminic acid has yet to be determined, tentative functions have been assigned which have identified PtmA to PtmH to be required for biosynthesis (PtmA, PtmB, PtmC, PtmD, PtmE, PtmF, PtmG, and PtmH are equivalent to the Cj1332, Cj1331, Cj1327, Cj1328, Cj1329, Cj1330, Cj1324, and Cj1325/6 proteins, respectively) (36). This *ptm* pathway is absent in *C. jejuni* strain 81-176. PtmG and PtmH from *C. coli* VC167 show 86 and 76% amino acid sequence similarity, respectively, to two hypothetical proteins, the Cj1324 and Cj1325/6 proteins of *C. jejuni* NCTC11168. The enzyme(s) involved in the attachment of glycan(s) to the flagellin protein of *Campylobacter* strains and the consensus sequence for the O-linked glycosylation process have yet to be identified.

In *C. jejuni* strain 81-176, glycosylation of flagellin has been shown to be necessary for the assembly of flagella and subsequent motility (19). There is extensive polymorphism in the *C. jejuni* O-linked glycosylation cluster, suggesting that selective pressure may cause the bacterium to alter surface antigens in attempts to evade the host immune defenses (59). Evidence supporting this possibility is demonstrated by comparing the glycan moieties of the flagella of *C. jejuni* 81-176 and *C. coli* VC167, as these strains produce unique modifications on their flagella which affect serospecificity (34).

Given the diversity of the O-linked glycosylation system in *C. jejuni* and the prevalence of the locus of Cj1321 to Cj1325/6 in chicken isolates, we hypothesized that these genes may be important for the abilities of some *C. jejuni* strains to colonize poultry and that colonization may be mediated through structural and surface charge changes in the glycan that modifies the flagellin. In this study, we demonstrate that Cj1324 is involved in the biosynthesis of two novel legionaminic acid modifications found on the flagellin of strain 11168H. The presence of these modifications affects autoagglutination, cell charge, and the efficiency with which *C. jejuni* 11168H colonizes chickens.

MATERIALS AND METHODS

Bacterial strains and growth media. All bacterial strains used in this study are shown in Table 1. *C. jejuni* strain 11168H is a hypermotile variant of the sequenced strain NCTC11168 that readily colonizes chickens (29). *C. jejuni* strains were cultured on Columbia blood agar (CBA) plates with Skirrow selective

TABLE 1. Strains used in this study

Strain or mutation[a]	Genotype or description	Reference or source
C. jejuni strains and mutations		
11168H	Hypermotile derivative of sequenced strain *C. jejuni* 11168	A. Karlyshev
ΔCj1321	ΔCj1321::Kan^r	This study
ΔCj1321 comp strain	ΔCj1321::Kan^r complement	This study
ΔCj1322	ΔCj1322::Kan^r	This study
ΔCj1322 comp strain	ΔCj1322::Kan^r complement	This study
ΔCj1323	ΔCj1323::Kan^r	This study
ΔCj1323 comp strain	ΔCj1323::Kan^r complement	This study
ΔCj1324	ΔCj1324::Kan^r	This study
ΔCj1324 comp strain	ΔCj1324::Kan^r complement	This study
ΔCj1325/6	ΔCj1325/6::Kan^r	This study
ΔCj1325/6 comp strain	ΔCj1325/6::Kan^r complement	This study
Δ(Cj1321-Cj1325/6)	Δ(Cj1321-Cj1325/6)::Kan^r	This study
Δ(Cj1321-Cj1325/6) comp strain	Δ(Cj1321-Cj1325/6)::Kan^r complement	This study
Δ*flaA*	Δ*flaA*::Kan^r	A. Karlyshev
11856	Livestock-associated clade strain	Campylobacter Reference Lab, United Kingdom
12450	Livestock-associated clade strain	Campylobacter Reference Lab, United Kingdom
12567	Livestock-associated clade strain	Campylobacter Reference Lab, United Kingdom
12241	Non-livestock-associated clade strain	Campylobacter Reference Lab, United Kingdom
15168	Non-livestock-associated clade strain	Campylobacter Reference Lab, United Kingdom
31467	Non-livestock-associated clade strain	Campylobacter Reference Lab, United Kingdom
43983	Non-livestock-associated clade strain	Campylobacter Reference Lab, United Kingdom
80646	Non-livestock-associated clade strain	Campylobacter Reference Lab, United Kingdom
E. coli strains		
Top10F′		Invitrogen (Paisley, United Kingdom)
XL-1		Stratagene
XL2-Blue MRF′		Stratagene

[a] Comp, complemented.

supplement (Oxoid, United Kingdom) and 7 to 9% horse blood in a variable-atmosphere incubator (VAIN; Don Whitley Scientific, United Kingdom) under microaerobic conditions (5% O_2, 85% N_2, and 10% CO_2) at 37°C for 24 or 48 h. Where necessary, CBA plates were supplemented with the antibiotics kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml). *C. jejuni* strains were stored at −80°C in a 12.5% solution of glycerol in Mueller-Hinton broth. *Escherichia coli* strains XL-1 (Stratagene, La Jolla, CA) and Top10 (Invitrogen, Paisley, United Kingdom) were used for cloning experiments and grown on Luria-Bertani agar or in Luria-Bertani broth at 37°C.

Electroporation. The electroporation procedure for *C. jejuni* strains was similar to the method described by Karlyshev and Wren (30). *C. jejuni* from glycerol stock was grown on CBA with the supplements listed above at 37°C for 48 h under microaerophilic conditions in a VAIN. Strains were restreaked and grown under the same conditions for 24 h. Growth from half a plate was resuspended in 1 ml of Mueller-Hinton broth, and the suspension was centrifuged at 13,000 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended and washed in 1 ml of ice-cold wash buffer (272 mM sucrose and 15% glycerol) and centrifuged at 13,000 rpm for 5 min. Cells were washed three times, and then 100-μl aliquots were used for each electroporation. After the addition of 1 to 5 μg of DNA, the mixture was transferred into an ice-cold electroporation cuvette and the cuvette was incubated on ice for 30 min. Electroporation was performed at 2.5 kV, 200 Ω, and 25 μF using the GenePulser Xcell system (Bio-Rad, Hemel Hempstead). The cuvette was flushed with 100 μl of superoptimal broth with catabolite repression (Sigma-Aldrich, United Kingdom), and cells were plated onto a nonselective CBA plate and grown with the plate upright for 24 h at 37°C. Bacteria were then plated onto selective plates and grown for a further 3 to 5 days.

Preparation of chromosomal DNA and general cloning techniques. Chromosomal DNA was extracted from *C. jejuni* by using a Puregene DNA purification kit per the instructions of the manufacturer (Gentra Systems). Purified DNA was stored at −20°C. Plasmid pRRC was used to complement all gene knockouts in *C. jejuni* strains. A description of the construction of and further details for this plasmid are given elsewhere (30).

Plasmids pUC18 and pUC19 (Fermentas, United Kingdom) were used for cloning. Plasmid DNA purification was performed using a Qiaprep spin miniprep kit (Qiagen, United Kingdom). Oligonucleotide primers were synthesized by Sigma-Aldrich Genosys Ltd. (Haverhill, United Kingdom). Restriction endonuclease enzymes and T4 DNA ligase were purchased from Promega (Southampton, United Kingdom). Standard restriction digestion, DNA ligation, and cloning procedures were as described elsewhere (48). Antarctic phosphatase was purchased from New England Biolabs (Ipswich, United Kingdom) and was used to dephosphorylate XbaI-treated pRRC according to the manufacturer's instructions.

Construction of ΔCj1321, ΔCj1322, ΔCj1323, ΔCj1324, and ΔCj1325/6 mutants. Site-specific mutations in *C. jejuni* Cj1322, Cj1324, and Cj1325/6 were generated via the insertion of a kanamycin resistance cassette (Kan^r) into unique sites present within appropriate pUC18-based recombinant plasmids from a random genomic library of *C. jejuni* (NCTC11168) that were constructed during the course of the genome-sequencing project (42) (Table 2). The Kan^r cassette was inserted in a nonpolar orientation, and the derivatives were used for the transformation of the *C. jejuni* 11168H strain via electroporation. Mutations were verified by PCR using gene-specific and kanamycin cassette-specific primers. ΔCj1321 and ΔCj1323 mutants were constructed by inverse PCR (IPCR) using plasmid cam169b1 from the NCTC11168 genome-sequencing library as a template (primer details are given in Table 3). The 1.9-kb insert in this plasmid contains full copies of Cj1321 and Cj1323. A BamHI fragment of plasmid pJMK30 containing the Kan^r cassette was blunt ended by T4 DNA polymerase. To construct the ΔCj1321 mutant, the IPCR product generated using primers ak338 and ak339 was blunt ended by T4 DNA polymerase and ligated to the Kan^r cassette. To construct the ΔCj1323 mutant, an IPCR product generated using

TABLE 2. pUC18-based plasmids used for mutagenesis

Gene mutated	Plasmid from pUC18 library	Insert size (kb)	Insertion site	Distance from gene start (% of gene size)
Cj1322	cam136d10	1.3	SwaI	77
Cj1324	cam19f3	1.3	EcoRV	34
Cj1325/6	cam165f10	1.9	ClaI	80

TABLE 3. PCR primers used for construction and verification of mutations ΔCj1321, ΔCj1323, and ΔCj1321-26 in 11168H and for construction of complementation vectors for ΔCj1324 and ΔCj1321-26 knockouts

Purpose and target gene(s)	Primer name	Sequence[a]
Mutagenesis		
Cj1321	ak338	GATAAAGATCTCTGCATAGTAAGCAGGTAGTATTATTAGC
	ak339	CGCTCAGATCTCTATGATGATGACATTTTTCACGTACTTC
	cj1320for	GGAAGCACCTATGAAAACAAGG
	cj1323rev	ATTTGCTAAAGCTCCTCGATTG
Cj1323	ak340	AAATAGATCTCTTTAAAAAGGCCTTATAAAAAGGAATTTTAG
	ak341	AAATAGATCTCGCAGTTTAAATCATCCATACTC
	cj1321for	AAAATGTCATCATCATAGGAGCG
	cj1324rev	TCTGCACACATTGTTCTATCCC
	ak240	GTGGTATGACATTGCCTTCTGCG
	ak241	TGGGTTTCAAGCATTAGTCCATGCAAG
Cj1321 to Cj1325/6	Cj1321cj1326locusF1	GCGGATCCGTATCGATAGCGGTTTTGTTT
	Cj1321cj1326locusR1	GCGCATGCAACCCACATCAAGCTTAAAAG
	inverse1321	GCGTTAACGATTTGTCCTACACCTAAAGC
	inverse1326	GCGTTAACCGATAAAGCCGAAGATCTAG
	puc19f	CTATGCGGCATCAGAGCAGATTG
Complementation		
Cj1324	cj1324for	GC**ACTAGT**AAGGAGAATTCTCATGATTTATTGTGATCACTGCGTGATGC
	cj1324rev	GC**ACTAGT**TTATTGTAATTTGTTTTTTAATTTAAATTCTC
Cj1321 to Cj1325/6	iacjlocusF	GC**ACTAGT**TAGGAGAAGTACGTCAAAAATGTCATCATCATAGG
	iacjlocusR	GC**ACTAGT**TTGCCTTTATTTTGCC

[a] Bases highlighted in bold indicate SpeI restriction sites, and underlined bases correspond to the SD sequence incorporated from the PorA gene.

primers ak340 and ak341 was blunt-ended by T4 DNA polymerase and ligated to a blunt-ended BamHI DNA fragment containing the Kan^r cassette. Following the transformation of *E. coli* XL2-Blue MRF′ cells, the recombinant plasmids were verified by restriction analysis and sequencing, and *C. jejuni* strain 11168H was transformed with the plasmids. The deletion of 94% of the gene target and the insertion of the Kan^r cassette into Cj1321 were confirmed by PCR using kanamycin gene-specific primers ak241 and ak240 and primers complementary to the adjacent genes Cj1320 and Cj1323. The deletion of 93% of the gene target and the insertion of the Kan^r cassette into Cj1323 were confirmed by PCR using kanamycin gene-specific primers ak241 and ak240 and primers complementary to the adjacent genes Cj1321 and Cj1324.

Construction of the Δ(Cj1321-Cj1325/6) mutant. PCR was used to amplify a ~5-kb region of DNA including 1,049 bp of sequence from the 3′ end of Cj1320 and 604 bp of sequence from the 5′ end of Cj1327 with primers Cj1321cj1326 locusF1 and Cj1321cj1326locusR1, the 5′ ends of which contained a BamHI and a SphI restriction site, respectively. Details of the primers used are listed in Table 3. The digested PCR product was cloned into BamHI- and SphI-digested pUC19 vector to produce pUC19locus. IPCR was used to generate a product which included 201 bp of DNA from the 5′ end of Cj1321 and 94 bp of DNA from the 3′ end of Cj1325/6, with the removal of the remaining sequence between Cj1321 and Cj1325/6 by using BIO-X-ACT long DNA polymerase from Bioline (London, United Kingdom) with primers inverse1321 and inverse1326 and pUC19locus as the template. The 5′ ends of primers inverse1321 and inverse1326 contained an HpaI restriction site. An HpaI-digested IPCR product was ligated with a kanamycin cassette that had been blunt ended with StuI to produce pUC19invlocus. A clone that contained Kan^r in the forward orientation was selected using primers puc19f and ak241, and this clone was introduced into 11168H by electroporation. A Δ(Cj1321-Cj1325/6) mutant was selected using CBA and kanamycin, and the mutation was confirmed by PCR and sequencing.

Complementation of ΔCj1324 and Δ(Cj1321-Cj1325/6). Cj1324 was amplified from *C. jejuni* 11168H by using *Pwo* high-fidelity *Taq* DNA polymerase (Roche, United Kingdom) for PCR (94°C for 2 min and 30 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 1.5 min, and 72°C for 5 min) with the primers listed in Table 3. SpeI restriction sites were included in the 5′ ends of the primers used to amplify Cj1324. To complement the Δ(Cj1321-Cj1325/6) mutant, the region from Cj1321 to Cj1325/6 was amplified using Accuprime high-fidelity *Taq* DNA polymerase (Invitrogen, United Kingdom) for PCR (95°C for 15 s and 34 cycles of 95°C for 15 s, 52°C for 15 s, and 68°C for 3 min) with the primers listed in Table 3. SpeI restriction sites were included in the 5′ ends of the primers used to amplify Cj1321 to Cj1325/6. In addition, Shine-Dalgarno (SD) sequences were incorporated into the forward primer upstream of the start codon for each gene where possible. For Cj1324, a native SD sequence could not be identified so the SD sequence corresponding to the gene encoding major outer membrane protein (PorA) was incorporated instead, as indicated in Table 3. To produce the delivery vectors, PCR fragments containing each of the genes were digested using SpeI and cloned into XbaI-digested pRRC in such a way that the genes would be transcribed in the same orientation as the chloramphenicol resistance gene, according to the method described in reference 30. Complementation vectors were sequenced and introduced into the appropriate mutants by electroporation. Complemented strains were selected using CBA plates containing kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml). The integration of Cm^r-Cj1324 and Cm^r-(Cj1321-Cj1325/6) gene fusions into the rRNA cluster on the 11168H chromosome was verified by PCR as described previously (30).

Autoagglutination assay. Autoagglutination assays were performed as described previously by Misawa and Blaser (39), with the modifications detailed below. Bacterial strains from glycerol stocks were grown on CBA or CBA plates supplemented with the appropriate antibiotics at 37°C for 48 h and then restreaked onto fresh plates and grown overnight. Growth from these plates was resuspended in phosphate-buffered saline (Sigma-Aldrich, United Kingdom), and the optical density at 600 nm (OD_{600}) was measured and adjusted to 1.0 ± 0.2. Two milliliters of each bacterial suspension was then dispensed into a sterile glass tube (13 by 100 mm), and the tubes were incubated microaerophilically at 37°C for 24 h in a VAIN. After incubation, 1 ml of the bacterial suspension from each glass tube was carefully removed and the OD_{600} was measured. Each strain was tested in triplicate. To ensure that any observed difference in phenotype was due to specific mutation, two separate clones for each of the ΔCj1321, ΔCj1324, and ΔCj1325/6 mutants were tested separately and then the data were combined. To account for the slight variations between the OD_{600}s of the strains tested, the data were normalized by subtracting the OD_{600} measured after 24 h from the starting OD_{600}, dividing by the starting OD_{600}, and finally multiplying by 100 to give the percent autoagglutination. Normalized results were plotted using GraphPad Prism version 4.02. Statistical significance was calculated using the Student *t* test, assuming equal variance. Results were considered to be statistically significant if P was <0.05.

Hydrophobicity assay. Hydrophobicity was determined by a salting-out method as described by Misawa and Blaser (39). The minimum concentration of ammonium sulfate allowing cells to aggregate defines the point of hydrophobicity. Sodium phosphate at 2 mM was used to make serial twofold dilutions of 4 M ammonium sulfate (25 μl each) to a final concentration of 0.00195 M in a U-bottomed 96-well plate. If a strain had not aggregated at the end point, its hydrophobicity was assigned as the next twofold dilution, 0.000975 M. Two-day-old cultures of *C. jejuni* were resuspended in 2 mM sodium phosphate, and the

OD_{600} was adjusted to approximately 1.0. Twenty-five microliters of the bacterial suspension was dispensed into each well, and the plate was incubated at 25°C for 24 h in a *Campylobacter* GasPak chamber with CampyGen packs (Oxoid, United Kingdom) to produce microaerophilic conditions. Each strain was tested in duplicate on at least two separate occasions. Clones 1 and 2 for ΔCj1321, ΔCj1322, ΔCj1323, ΔCj1324, and ΔCj1325/6 were tested separately and then the data were combined. Statistical significance was calculated using a two-tailed Mann-Whitney U test. Results were considered to be statistically significant if P was <0.05.

Biofilm assay. Biofilm assays were set up using glass tubes (13 by 100 mm) as described previously (25). Two-day-old bacterial cultures were resuspended in *Brucella* broth to an OD_{600} of ~0.04 on average, and 5 ml was dispensed into each tube. The tubes were incubated microaerophilically at 37°C for 7 days without shaking. Each strain was tested in triplicate on three separate occasions.

Adhesion and invasion assays. Procedures for the infection of primary chick kidney cells (CKC) and HD11 avian macrophage-like cells with *C. jejuni* have been described previously (55). The adherence of wild-type and mutant strains was evaluated at 1 h postinoculation of semiconfluent monolayers at a multiplicity of infection of 100:1 by repeated washing of monolayers with prewarmed phosphate-buffered saline, followed by lysis with 0.5% (vol/vol) Triton X-100 and the plating of serial 10-fold dilutions of lysates onto sheep blood agar. Intracellular bacteria were enumerated as described above following the incubation of washed monolayers with gentamicin at a final concentration of 100 μg/ml for 1 h (55). Triplicate determinations within a single assay were performed, and the data represent the mean ± standard deviation for three independent biological replicates of each assay. Similarly, the procedure for quantifying the adhesion of *C. jejuni* to human Hep2 cells was performed as described previously (28).

Top-down mass spectrometry (MS) analysis of flagellin. Flagellin was purified as described previously in reference 13 and was then dialyzed in H_2O (with 0.2% formic acid) by using a Centricon YM-30 membrane filter. The flagellin was concentrated to 0.2 mg/ml and infused into a Waters quadrupole time-of-flight mass spectrometer at a flow rate of 0.5 μl/min. Multiply protonated flagellin precursor ions were subjected to top-down analysis according to the method described in reference 50.

Metabolomic analysis of *C. jejuni* strains by hydrophilic interaction liquid chromatography (HILIC)-MS. Cell lysates from overnight 500-ml cultures of *C. jejuni* strains and 11168H mutant strains were probed for intracellular sugar nucleotides as described previously (51). The extraction of sugar nucleotide sugars was achieved using Envi-Carb solid-phase extraction cartridges as described previously (37).

Experimental animals. Animal experiments were performed in accordance with the Animals (Scientific Procedures) Act of 1986 under PPL 30/2462. Specific-pathogen-free outbred Light Sussex chickens were produced at the Institute for Animal Health, Compton, United Kingdom. On the day of hatching, chickens orally received 0.1 ml of a *Campylobacter*-free adult gut flora preparation (23). Inoculated birds were housed in separate rooms in a high-biosecurity facility until they were 2 weeks old, after which they were used in colonization trials. Birds were given access to water and a vegetable-based diet (Special Diet Services, Manea, Cambridgeshire, United Kingdom) ad libitum.

Colonization trials. Groups of 10 2-week-old birds with developed gut floras were separately dosed with ca. 10^7 CFU of the 11168H wild type or a mutant or complemented strain by oral gavage. Inocula were confirmed to be comparable by retrospective viable cell count determination. The magnitude and duration of excretion were monitored at weekly intervals for 6 weeks by a semiquantitative method reliant on the direct plating of cloacal swabs onto *Campylobacter* blood-free selective agar, with incubation in a microaerobic atmosphere at 37°C for 48 h (56). The swabs were also incubated for 48 h in enrichment broth at 37°C and then plated onto *Campylobacter* blood-free selective agar and scored for the presence of *Campylobacter* bacteria; the resulting counts were referred to as enrichment counts. At the end of the trial, postmortem examinations were performed to enumerate viable bacteria in the intestinal contents at several points along the intestinal tract. Contents were collected, weighed, and resuspended in phosphate-buffered saline to a final concentration of 0.1 g of cecal contents per ml. Tenfold serial dilutions of each sample were made and plated onto blood-free medium or medium supplemented with kanamycin (30 μg/ml) or kanamycin (30 μg/ml) and chloramphenicol (10 μg/ml) to select for mutant or complemented strains, respectively. The theoretical limit of detection by this method is 100 CFU/g. Levels of bacteria recovered from different intestinal sites were analyzed for the effect of the strain by using a two-way analysis with a general linear model (PROC GLM) after $\log_{10}$ transformation with the Statistical Analysis System, 1995 (SAS Institute, Cary, NC). Pairwise comparisons were performed using least-squares mean differences at the overall 0.05 level of significance.

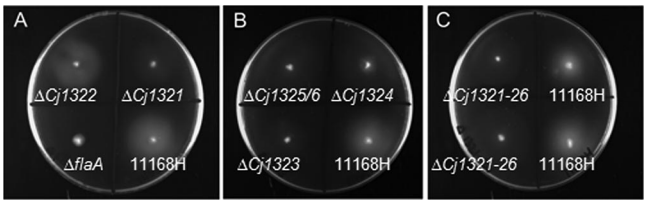

FIG. 1. Swimming motility is not affected by the mutation of Cj1321, Cj1322, Cj1323, Cj1324, Cj1325/6, or Cj1321 to Cj1325/6. Motility assays were performed using 0.4% Mueller-Hinton agar inoculated with strains with the indicated genotypes or strain designations and incubated for 48 h under microaerophillic conditions. The flagellin-negative Δ*flaA* mutant was also included as a negative control.

RESULTS

Prevalence of Cj1321, Cj1322, Cj1323, Cj1324, and Cj1325/6 in *Campylobacter* spp. The strains used in this study are summarized in Table 1. Amino acid sequences predicted from the Cj1321-Cj1325/6 locus were used to investigate the presence of similar homologues in other species by BlastX analysis (17). Degrees of amino acid identity to the Cj1321, Cj1322, Cj1323, Cj1324, and Cj1325/6 proteins of *C. jejuni* 11168H were calculated using EMBOSS with Smith-Waterman local alignment (46, 57). Orthologues of Cj1321, Cj1322, and Cj1323 were found in *C. jejuni* subsp. *jejuni* 84-25 and *C. jejuni* subsp. *jejuni* CF93-6. Cj1324 and Cj1325/6 were found in larger numbers of strains. Cj1324 was present in *C. jejuni* CF93-6, *C. jejuni* 84-25, *Campylobacter upsaliensis* RM3195, and *C. coli* VC167. Cj1325/6 was identified in *C. coli* VC167, *C. upsaliensis* RM3195, *Campylobacter lari* RM2100, *C. jejuni* 84-25, *C. jejuni* CF93-6, and *C. jejuni* subsp. *doylei* 269.97.

Mutation of Cj1321, Cj1322, Cj1323, Cj1324, and Cj1325/6 does not affect motility. *C. jejuni* strain 11168H was chosen for the investigation of the functions of genes within the locus of Cj1321 to Cj1325/6. Site-directed mutagenesis was used to generate knockout mutations of each of the genes individually by inserting a kanamycin resistance cassette. To ensure that any differences in phenotypic traits were due to specific mutations in the gene of interest, two independent knockouts (clones 1 and 2) for each gene were generated. In addition, the whole locus (Cj1321 to Cj1325/6) was disrupted by allelic replacement with a kanamycin resistance cassette. ΔCj1324 and Δ(Cj1321-Cj1325/6) were complemented using the method described by Karlyshev and Wren (30). Previous studies have shown that the mutation of a number of genes within the flagellar glycosylation island can result in the loss of bacterial motility (19, 21, 62). The swimming motilities of 11168H and the ΔCj1321, ΔCj1322, ΔCj1323, ΔCj1324, ΔCj1325/6, and Δ(Cj1321-Cj1325/6) mutants were tested using 0.4% Mueller-Hinton agar plates (11168H Δ*flaA* was included as a negative control). All strains displayed swimming motility, as shown in Fig. 1. In addition, strain 11168H and the ΔCj1324 and Δ(Cj1321-Cj1325/6) mutants were examined by electron microscopy using negative staining and showed no observable differences in flagella (Fig. 2). Thus, mutations in genes Cj1321 to Cj1325/6 do not affect the assembly of the flagellum or the motility of the organism.

Cj1324 is involved in autoagglutination and alters hydrophobicity in *C. jejuni* 11168H. The association between auto-

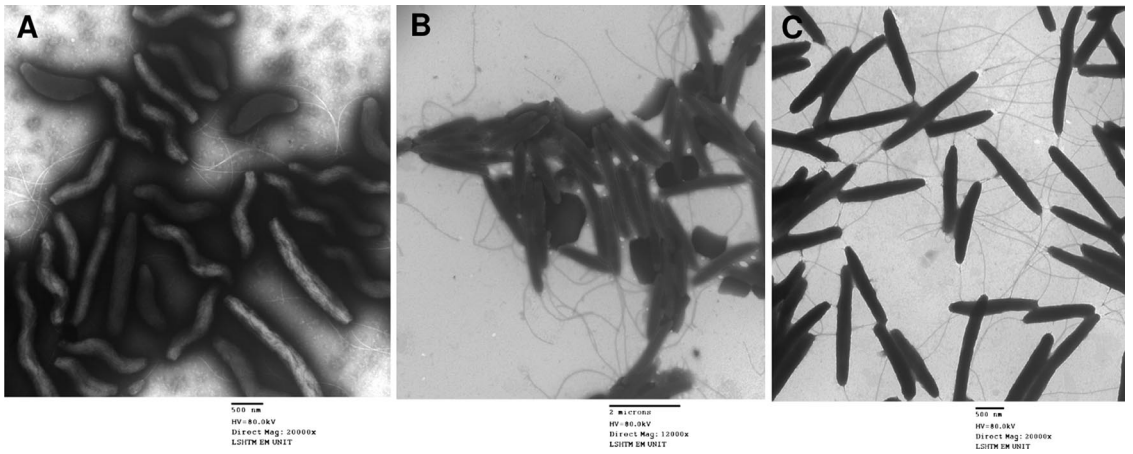

FIG. 2. Transmission electron microscopy analysis of *C. jejuni* cells. Flagellum production is not affected by the mutation of Cj1324 and Cj1321 to Cj1325/6. The strains examined were as follows: wild-type strain 11168H (A), the ΔCj1324 mutant (B), and the Δ(Cj1321-Cj1325/6) mutant (C). Samples were fixed in 2.5% glutaraldehyde–2.5% paraformaldehyde–0.1 M sodium cacodylate buffer, pH 7.4, placed on a grid, and negatively stained for 1 min with 2% aqueous uranyl acetate before being examined with a Joel 1200EX transmission electron microscope at 80 kV.

agglutination and virulence for many pathogenic gram-negative bacteria, including *Neisseria gonorrhoeae* (58) and *Bordetella pertussis* (38), has been described previously. In *C. jejuni*, autoagglutination is likely to be controlled by multiple factors. However, it has been demonstrated that the presence of FlaA (39) and, more specifically, posttranslational modifications of flagellin are required for autoagglutination (21, 39). Given that genes Cj1321 to Cj1325/6 are located within the flagellin glycosylation locus, 11168H and the ΔCj1321, ΔCj1322, ΔCj1323, ΔCj1324, ΔCj1325/6, and Δ(Cj1321-Cj1325/6) mutants were tested for their abilities to autoagglutinate. In addition, the flagellin-negative mutant 11168H Δ*flaA* was included as a control. The Δ*flaA* strain exhibited dramatically reduced autoagglutination compared to that of the wild type, forming a cloudy suspension after 24 h (Fig. 3), as reported previously by Misawa and Blaser (39). The autoagglutination levels of the ΔCj1321, ΔCj1322, ΔCj1323, and ΔCj1325/6 strains were comparable to that of the wild-type strain 11168H, which was approximately 90% after 24 h (data are shown for the ΔCj1321, ΔCj1325/6, and 11168H strains). In contrast, both the ΔCj1324 and Δ(Cj1321-Cj1325/6) mutants had significantly lower levels of autoagglutination, 54 and 60%, respectively, than the wild-type strain 11168H after 24 h ($P < 0.0001$). Autoagglutination levels were restored to levels comparable to that of 11168H for both the ΔCj1324 and Δ(Cj1321-Cj1325/6) strains when the mutations were complemented by the insertion of the respective wild-type alleles into the chromosome (Fig. 3).

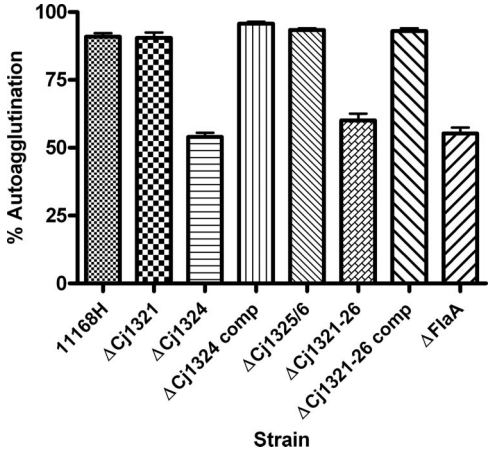

FIG. 3. ΔCj1324, Δ(Cj1321-Cj1325/6), and Δ*flaA* mutations impair autoagglutination by *C. jejuni* 11168H. The bacterial strains specified by genotype or strain designation along the *x* axis were resuspended to an OD_{600} of approximately 1.0 in phosphate-buffered saline, and the suspensions were incubated at 37°C under microaerophillic conditions without shaking. The percent autoagglutination was calculated as described in Materials and Methods. comp, complemented strain.

Changes in autoagglutination abilities are strongly associated with alterations to hydrophobicity (39). Gene-specific knockouts in the ΔCj1321, ΔCj1322, ΔCj1323, ΔCj1324, ΔCj1325/6, and Δ(Cj1321-Cj1325/6) strains were tested using the salting-out method to determine the hydrophobicity of each strain. As shown in Fig. 4, 11168H was strongly hydrophobic, aggregating at an average concentration of 0.01 M ammonium sulfate. Gene knockouts in the ΔCj1321, ΔCj1322, ΔCj1323, and ΔCj1325/6 strains did not reduce the hydrophobicity of these strains. In contrast, the ΔCj1324 mutation resulted in a significant reduction in hydrophobicity compared to that of the wild type, with cells aggregating at an average concentration of 0.09 M ammonium sulfate ($P < 0.0001$). This reduction in hydrophobicity was eliminated when the mutant strain was complemented. Further evidence suggesting that Cj1324 may play a role in altering hydrophobicity was seen in the Δ(Cj1321-Cj1325/6) mutant, which also showed a significant decrease in hydrophobicity, with aggregation at an average of 0.09 M ($P < 0.0001$). Previous research has shown that the ability of *C. jejuni* to autoagglutinate can affect both adherence to and invasion of intestinal epithelial cells (18, 21, 39). Given the differences in autoagglutination and hydrophobicity observed for the ΔCj1324 and Δ(Cj1321-Cj1325/6) mutants compared to 11168H, we tested the abilities of these strains to adhere to and invade primary CKC and an avian

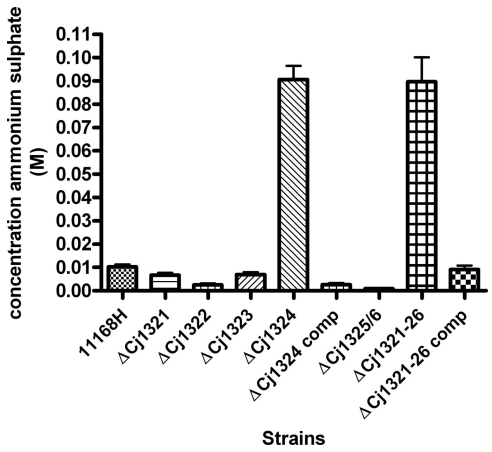

FIG. 4. ΔCj1324 and Δ(Cj1321-Cj1325/6) mutations reduce the surface hydrophobicity of *C. jejuni* 11168H. Hydrophobicity was measured by determining the minimum concentration of ammonium sulfate that permitted the aggregation of the bacterial strains specified by genotype or strain designation along the *x* axis by using serial twofold dilutions of a 4 M solution. The end point concentration for this assay was 0.00195 M ammonium sulfate. comp, complemented strain.

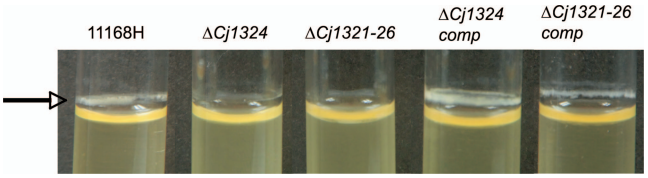

FIG. 5. ΔCj1324 and Δ(Cj1321-Cj1325/6) mutations impair biofilm formation. Biofilm assays were performed using 2-day-old cultures resuspended in 5 ml of *Brucella* broth to an OD_{600} of ~0.04 and incubated microaerophilically at 37°C for 7 days without shaking. The arrow denotes the position of the biofilm on the glass surface above the meniscus.

macrophage-like cell line (HD11) and adhere to human cervix carcinoma (Hep2) cells. The mean levels of viable bacteria recovered ± standard errors (in $\log_{10}$ CFU per milliliter) are as follows: for bacteria adhering to CKC, 5.02 ± 0.71 (11168H), 4.97 ± 0.66 (ΔCj1324 mutant), and 5.00 ± 0.47 [Δ(Cj1321-Cj1325/6) mutant]; for bacteria adhering to HD11 cells, 5.93 ± 0.44 (11168H), 5.43 ± 0.34 (ΔCj1324 mutant), and 5.47 ± 0.77 [Δ(Cj1321-Cj1325/6) mutant]; for bacteria adhering to Hep2 cells, 4.23 ± 0.24 (11168H), 4.43 ± 0.24 (ΔCj1324 mutant), and 4.27 ± 0.43 [Δ(Cj1321-Cj1325/6) mutant]; for bacteria invading CKC, 4.59 ± 0.35 (11168H), 4.21 ± 0.64 (ΔCj1324 mutant), and 4.10 ± 0.71 [Δ(Cj1321-Cj1325/6) mutant]; and for bacteria invading HD11 cells, 4.89 ± 0.74 (11168H), 5.08 ± 0.73 (ΔCj1324 mutant), and 5.15 ± 0.83 [Δ(Cj1321-Cj1325/6) mutant]. *P* values were >0.05 by pairwise analysis of variance, indicating that differences were not significant.

Biofilm formation by the ΔCj1324 and Δ(Cj1321-Cj1325/6) mutants is reduced. Several studies have demonstrated that *C. jejuni* can form biofilms on abiotic surfaces (25, 26, 44). As flagella have recently been shown to affect the formation of biofilms by *C. jejuni* (44), assays to determine whether Cj1324 and Cj1321 to Cj1325/6 are involved in this trait were performed. Biofilms approximately 2 to 3 mm wide attached to the walls of glass tubes were clearly visible when 11168H was tested, as shown in Fig. 5. Previous studies have confirmed that this observation by electron microscopy is indicative of a mature biofilm for the wild-type strain 11168H (25). For both the ΔCj1324 and Δ(Cj1321-Cj1325/6) mutants, there was a significant reduction in biofilm formation compared to that by the wild type after 7 days. When ΔCj1324 and Δ(Cj1321-Cj1325/6) were complemented, biofilm formation was restored. There were no observed differences in biofilm formation between the ΔCj1321, ΔCj1322, ΔCj1323, or ΔCj1325/6 mutant and 11168H (data not shown).

Colonization of chick ceca by ΔCj1324 and Δ(Cj1321-Cj1325/6) mutants is reduced. *C. jejuni* 11168H and the isogenic ΔCj1324 and Δ(Cj1321-Cj1325/6) mutants were separately administered to outbred chickens via the oral route, and the magnitude and duration of excretion were monitored by semiquantitative direct plating and the enrichment of cloacal swabs taken at weekly intervals. 11168H colonized 100% of birds for the first 5 weeks, as determined by the enrichment of cloacal swabs and in some instances direct plating (Fig. 6A and

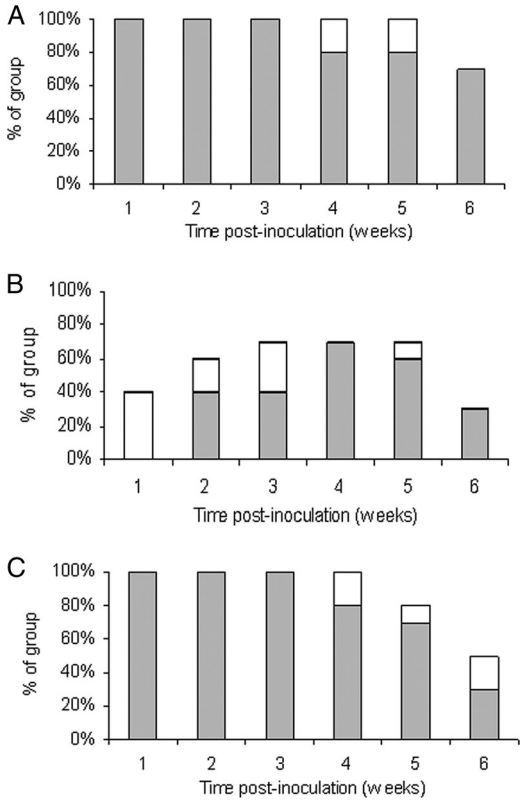

FIG. 6. Magnitude and duration of excretion of *C. jejuni* 11168H (A), the Δ(Cj1321-Cj1325/6) mutant (B), and the Δ(Cj1321-Cj1325/6) complemented strain (C) as determined by semiquantitative analyses of cloacal swabs taken at weekly intervals after the oral inoculation of outbred chickens. Dark gray bars indicate the percentage of each group with direct counts of *C. jejuni* bacteria. White bars indicate the percentage of each group in which the presence of *Campylobacter* was detected only upon enrichment. Birds classified as positive by direct plating would also be positive by enrichment culture.

TABLE 4. Recovery of viable bacteria of *C. jejuni* 11168H, ΔCj1321-26 mutant, and ΔCj1321-26 complemented strains from the gastrointestinal tracts of chickens[a]

Site in gastrointestinal tract	Log no. of CFU of:		
	11168H	ΔCj1321-26 mutant	ΔCj1321-26 complemented strain
Crop	2.9 ± 0.14	2.0 ± 0.06[b]	2.9 ± 0.17
Gizzard	2.7 ± 0.24	2.2 ± 0.04[b]	2.6 ± 0.11
Jejunum	2.6 ± 0.24	2.0 ± 0.07[b]	3.2 ± 0.19
Ileum	4.0 ± 0.34	3.3 ± 0.04[c]	3.7 ± 0.21
Cecum	7.3 ± 0.23	5.7 ± 0.47[b]	8.0 ± 0.12
Cecal tonsils	7.1 ± 0.13	5.6 ± 0.25[b]	6.8 ± 0.19

[a] Samples were recovered 6 weeks postinoculation (10 birds per strain). Log values presented are the geometric mean CFU per gram ± the standard errors of the means.

[b] Differences from data for both the parent and the complemented strain are statistically significant ($P < 0.05$).

[c] The difference from data for the parent is statistically significant ($P < 0.05$). Data for the complemented strain are not statistically significantly different from those for either the parent or the mutant.

7A), and extensive colonization of distal intestinal sites was detected by viable cell count determination at postmortem examination 6 weeks after challenge (Tables 4 and 5). Mean levels of bacteria recovered from 10 birds per group at postmortem examination are presented, and disparity between these results and the findings from enrichment culture of cloacal swabs reflects the relative insensitivity of the latter approach compared to that of direct plating of intestinal contents. Among Δ(Cj1321-Cj1325/6) mutant-inoculated chickens, 40% of birds were colonized in the first week postinoculation and 60% of birds (40% detected by direct counting and 20% detected by enrichment culture) were colonized at 2 weeks postinoculation (Fig. 6B). The highest level of colonization detected in birds inoculated with the Δ(Cj1321-Cj1325/6) mutant was 70%, during weeks 3, 4, and 5, after which the mutant began to be cleared from the birds. The colonization of chickens to levels comparable to those of colonization with 11168H was observed when Δ(Cj1321-Cj1325/6) was complemented (Fig. 6C). Similarly, in the case of the ΔCj1324 mutant, 40% of chickens were colonized in both the first and second weeks (Fig. 7B) and levels of colonization reached 70% during weeks

TABLE 5. Recovery of viable bacteria of *C. jejuni* 11168H, ΔCj1324 mutant, and ΔCj1324 complemented strains from the gastrointestinal tracts of chickens[a]

Site in gastrointestinal tract	Log no. of CFU of:		
	11168H	ΔCj1324 mutant	ΔCj1324 complemented strain
Crop	2.7 ± 0.2	2.6 ± 0.12	2.7 ± 0.14
Gizzard	2.5 ± 0.06	2.3 ± 0.08	2.5 ± 0.12
Jejunum	2.4 ± 0.1	2.3 ± 0.1	2.7 ± 0.1
Ileum	3.5 ± 0.12	3.3 ± 0.17	3.7 ± 0.19
Cecum	8.6 ± 0.13	7.4 ± 0.19[b]	8.3 ± 0.08
Cecal tonsils	6.3 ± 0.13	5.5 ± 0.25[b]	6.2 ± 0.08

[a] Samples were recovered 6 weeks postinoculation (10 birds per strain). Log values presented are the geometric mean CFU per gram ± the standard errors of the means.

[b] Differences from data for both the parent and the complemented strain are statistically significant ($P < 0.05$).

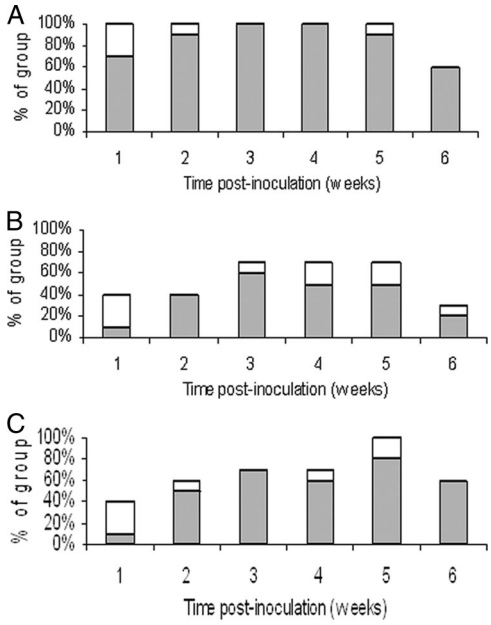

FIG. 7. Magnitude and duration of excretion of *C. jejuni* 11168H (A), the ΔCj1324 mutant (B), and ΔCj1324 complemented strain (C) as determined by semiquantitative analyses of cloacal swabs taken at weekly intervals after the oral inoculation of outbred chickens. Dark gray bars indicate the percentage of each group with direct counts of *C. jejuni* bacteria. White bars indicate the percentage of each group in which the presence of *C. jejuni* was detected only upon enrichment. Birds classified as positive by direct plating would also be positive by enrichment culture.

3, 4, and 5, after which the ΔCj1324 mutant began to be cleared. The ΔCj1324 complemented strain also appeared to be cleared during weeks 3, 4, and 5 but not to the same extent as the ΔCj1324 mutant. The numbers of viable bacteria in the crop, gizzard, jejunum, ileum, and ceca at 6 weeks postinoculation with the Δ(Cj1321-Cj1325/6) and ΔCj1324 mutants relative to those at 6 weeks postinoculation with the parent and complemented strains are recorded in Tables 4 and 5. The Δ(Cj1321-Cj1325/6) and ΔCj1324 mutant bacteria were recovered from the cecal contents and cecal tonsils in significantly lower numbers than bacteria of the parent strain ($P < 0.05$). In addition, all sites of the gastrointestinal tract had significantly lower levels of colonization with the Δ(Cj1321-Cj1325/6) mutant than with 11168H (Table 4) ($P < 0.05$). The numbers of viable bacteria of the Δ(Cj1321-Cj1325/6) complemented and ΔCj1324 complemented strains were comparable to those of the wild type and statistically significantly different from those of the corresponding mutants at sites where attenuation in colonization by the mutant strains was detected except the ileum (Tables 4 and 5), indicating that the attenuation of the mutant strains is unlikely to be a consequence of secondary defects. Analysis of the data by a Wilcoxon Mann-Whitney nonparametric test confirmed the statistically significant attenuation of the ΔCj1324 and Δ(Cj1321-Cj1325/6) strains detected by analysis-of-variance F tests. We also tested the colonization of chickens by the ΔCj1321, ΔCj1322, ΔCj1323, and ΔCj1325/6 mutants compared to that by 11168H. There were no observed differences in the colonization of chickens between the ΔCj1321, ΔCj1322, ΔCj1323, or ΔCj1325/6 mutant and 11168H. Also, no significant differences in colonization

TABLE 6. Recovery of viable bacteria of *C. jejuni* 11168H, ΔCj1321 mutant, ΔCj1322 mutant, ΔCj1323 mutant, and ΔCj1325 mutant strains and corresponding complemented strains from the gastrointestinal tracts of chickens[a]

Mutation	Site in gastrointestinal tract	Log no. of CFU of:		
		11168H	Deletion mutant	Complemented strain
ΔCj1321	Crop	2.39 ± 0.75	2.30 ± 0.73	2.37 ± 0.48
	Gizzard	2.35 ± 0.40	2.31 ± 0.83	2.73 ± 0.41
	Jejunum	2.46 ± 0.63	2.40 ± 0.62	2.62 ± 0.30
	Ileum	3.39 ± 0.41	3.35 ± 0.18	3.64 ± 0.67
	Cecum	7.42 ± 0.51	7.51 ± 0.52	7.70 ± 0.91
	Cecal tonsils	6.53 ± 0.62	6.60 ± 0.91	6.47 ± 0.84
ΔCj1322	Crop	2.39 ± 0.75	2.28 ± 0.88	2.46 ± 0.72
	Gizzard	2.35 ± 0.40	2.33 ± 0.13	2.40 ± 0.45
	Jejunum	2.46 ± 0.63	2.32 ± 0.85	2.43 ± 0.20
	Ileum	3.39 ± 0.41	3.30 ± 0.65	3.50 ± 0.97
	Cecum	7.42 ± 0.51	7.55 ± 0.77	7.48 ± 0.81
	Cecal tonsils	6.53 ± 0.62	6.46 ± 0.51	6.40 ± 0.63
ΔCj1323	Crop	2.39 ± 0.75	2.34 ± 0.58	2.29 ± 0.13
	Gizzard	2.35 ± 0.40	2.40 ± 0.17	2.30 ± 0.74
	Jejunum	2.46 ± 0.63	2.41 ± 0.22	2.44 ± 0.24
	Ileum	3.39 ± 0.41	3.30 ± 0.76	3.31 ± 0.15
	Cecum	7.42 ± 0.51	7.37 ± 0.59	7.30 ± 0.66
	Cecal tonsils	6.53 ± 0.62	6.49 ± 0.88	6.67 ± 0.75
ΔCj1325	Crop	2.39 ± 0.75	2.48 ± 0.25	2.65 ± 0.90
	Gizzard	2.35 ± 0.40	2.53 ± 0.29	2.38 ± 0.60
	Jejunum	2.46 ± 0.63	2.60 ± 0.89	2.35 ± 0.78
	Ileum	3.39 ± 0.41	3.58 ± 0.80	3.43 ± 0.72
	Cecum	7.42 ± 0.51	7.49 ± 0.28	7.56 ± 0.92
	Cecal tonsils	6.53 ± 0.62	6.50 ± 0.68	6.61 ± 0.36

[a] Samples were recovered 6 weeks postinoculation (10 birds per strain). Log values presented are the geometric mean CFU per gram ± the standard errors of the means.

between these mutants and 11168H at any sites of the gastrointestinal tract were found (Table 6).

Metabolomic analysis of 11168H and the ΔCj1324 mutant. To investigate the effect of insertional inactivation on the production of flagellar glycans, we examined the CMP sugar nucleotide compositions of cell lysates from *C. jejuni* 11168H and the corresponding ΔCj1324 mutant. By using HILIC-MS and precursor ion scanning for fragment ions characteristic of CMP, cell lysates from 11168H were found to contain an intracellular pool of CMP-linked sugars (Table 7). CMP-linked sugars with retention times corresponding to those of the previously characterized nonulosonate sugars Pse5Ac7Ac (316 Da), Pse5Ac7Am (315 Da), Leg5Ac7Ac (316 Da), Leg5 Am7Ac (315 Da), and Leg5AmNMe7Ac (330 Da) were present in the metabolome. CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac), which correlates with the presence of Neu5Ac in the lipooligosaccharide (LOS) from this strain, was also present. In addition, precursor ion scanning experiments revealed two novel CMP intermediates as negative ions with *m/z* values of 711 and 712, with corresponding glycans with masses of 389 and 390 Da. These glycans were recently shown to be the novel 2,3-di-*O*-methylglyceric acid derivatives of pseudaminic acid and are present on the flagellin of *C. jejuni* 11168 (33). Metabolomic analysis of the ΔCj1324 mutant revealed the disappearance of CMP-Leg5Am7Ac and CMP-Leg5AmNMe7Ac (Table 7), as well as CMP-Pse5Ac7Am. Complementation of the mutant with the Cj1324 gene restored both CMP-Leg5Am7Ac and CMP-Leg5AmNMe7Ac but not CMP-Pse5Ac7Am. This result was consistent for three independent clones of the ΔCj1324 mutant and three corresponding complementation strains. The loss of CMP-Pse5Ac7Am in the metabolomes of the ΔCj1324 mutant and the ΔCj1324 complemented strain may be a consequence of increased incorporation of the other Pse derivatives into the flagellin structural proteins, thus diminishing levels of the nucleotide-activated precursor in the cytosol. This effect would occur as a consequence of the inability to synthesize either of the CMP-legionaminic acid derivatives. The inability to restore a wild-type profile in complementation experiments may be a reflection of subtle pathway cross talk/regulatory mechanisms which we do not yet understand (e.g., pathway intermediate accumu-

TABLE 7. HILIC-MS analysis of CMP intracellular sugar nucleotides[a]

Strain or mutation	Presence of:							
	CMP intermediate (*m/z* 712; 13.1 min)	CMP-Leg5Ac7Ac (*m/z* 638; 14.1 min)	CMP-Pse5Ac7Ac (*m/z* 638; 14.4 min)	CMP-Neu5Ac (*m/z* 613; 15.7 min)	CMP intermediate (*m/z* 711; 16.9 min)	CMP-Leg5AmNMe7Ac (*m/z* 651; 17.7 min)	CMP-Leg5Am7Ac (*m/z* 637; 18.4 min)	CMP-Pse5Ac7Am (*m/z* 637; 19.2 min)
11168H	+	+	+	+	+	+	+	+
ΔCj1324	+	+	+	+	+	−	−	−
ΔCj1324 comp strain	+	+	+	+	+	+	+	−
ΔCj1321-26	+	+	+	+	+	−	−	+
ΔCj1321-26 comp strain	+	+	+	+	+	+	+	+
11856[b]	+	+	+	+	+	+	+	+
12450[b]	+	+	+	+	+	+	+	+
12567[b]	+	+	+	+	+	+	+	+
12241[c]	+	+	+	+	+	−	−	+
15168[c]	+	+	−	−	+	−	−	+
31467[c]	+	+	−	−	+	−	−	−
43983[c]	+	+	−	+	+	−	−	+
80646 (Cj1324$^+$)[d]	−	+	+	−	−	−	+	+

[a] Values in minutes are retention times. Comp, complemented; −, sugar nucleotide not detected; +, sugar nucleotide detected.
[b] Livestock-associated clade.
[c] Non-livestock-associated clade.
[d] The Cj1324$^+$ gene is present in this non-livestock-associated strain.

lation and feedback regulation) or may be due to a different level of gene expression in the complemented strain. For the whole-locus knockout Δ(Cj1321-Cj1325/6), both CMP-Leg5 Am7Ac and CMP-Leg5AmNMe7Ac disappeared, but these CMP-linked sugars were restored upon complementation.

Flagellin protein structural analysis. Because the mutation of the Cj1321-Cj1325/6 locus did not affect flagellin protein production, we were able to undertake top-down MS analysis of purified flagellin protein from *C. jejuni* 11168H and the corresponding ΔCj1324 mutant. Analysis of *C. jejuni* 11168H flagellin confirmed the presence of monosaccharides corresponding to each of the CMP-linked nucleotide sugars observed in the metabolome. Carbohydrate moieties with masses of 315, 316, 389, and 390 Da were all present on 11168H flagellin protein. We were unable to observe the 329-Da modification on the flagellin protein by top-down analysis, due most likely to the low abundance of this glycan modification relative to those of the other flagellar modifications. It should also be noted that due to identical masses, it is not possible to distinguish Pse5Ac7Am from Leg5Am7Ac (315 Da) or Pse5Ac7Ac from Leg5Ac7Ac (316 Da) by this type of analysis. In contrast, flagellin from the ΔCj1324 mutant lacked the 315-Da glycan yet still contained 389-, 390-, and 317-Da glycans. Complementation of ΔCj1324 resulted in flagellin which carried the 315-Da glycan.

Metabolomic analysis of livestock-associated and non-livestock-associated clade strains. To determine if the ability to synthesize the two legionaminic acid derivatives was also present in other isolates, we next examined the metabolomes of a number of *C. jejuni* strains that had been classified previously by comparative phylogenomic analysis as belonging to the livestock-associated or non-livestock-associated clade. Three livestock-associated clade isolates, 11856, 12450, and 12567, which contained the Cj1321-Cj1325/6 locus, all produced CMP-Leg5Am7Ac and CMP-Leg5AmNMe7Ac, in addition to all the other CMP-activated sugars found in the metabolome of 11168H (Table 7). In contrast, the metabolomes of the four tested non-livestock-associated clade strains (12241, 15168, 31467, and 43983), in which Cj1324 was absent, all lacked the two novel CMP-Leg derivatives (Table 7). The metabolomes of two of these strains, 15168 and 31467, were also lacking CMP-Neu5Ac. Neu5Ac is a component of LOS in many strains of *C. jejuni*, and microarray analysis confirmed that these strains lacked the sialic acid biosynthetic genes to synthesize the CMP precursor (by microarray analysis, genes Cj1136 to Cj1145 were found to be absent in strain 15168 and genes Cj1135 to Cj1145 were found to be absent in strain 31467). A non-livestock-associated clade strain, 80646, which contained the Cj1324 gene, appeared to still make CMP-Leg5 Am7Ac but not CMP-Leg5AmNMe7Ac (microarray analysis confirmed that this strain lacks Cj1325/6). In addition, the metabolome of 80646 appeared to lack the two novel CMP-Pse 2,3-di-*O*-methylglyceric acid derivatives as negative ions with *m*/*z* values of 711 and 712.

DISCUSSION

In a previous study using whole-genome comparative phylogenomic analyses of *C. jejuni*, we demonstrated that isolates fall into two distinct groups, a livestock-associated clade, which contained 31 of 35 livestock strains analyzed, and a non-livestock-associated clade (10). The study identified genes associated with strains of the livestock-associated clade, one of the most prominent being a cluster of five genes (Cj1321 to Cj1325/6) adjacent to the structural flagellin genes. Cj1321 to Cj1325/6 are located within the flagellar glycosylation locus, a highly variable region of the *C. jejuni* genome which ranges in size from ~21 to 50 genes. These findings, together with the location of Cj1321 to Cj1325/6 within strain 11168H, led us to explore the role these genes may play in flagellum glycosylation in *C. jejuni*.

It is becoming increasingly apparent that the glycosylation of flagella of gram-negative bacteria is important, with glycosylation modifications increasing the hydrophobicity of flagellin and often influencing the bacterium's immunogenicity, interaction with eukaryotic cells, and possibly, evasion of natural predators (9, 51). For *Campylobacter*, most reports to date characterizing the O-linked glycosylation of flagellin have focused on *C. coli* VC167 and *C. jejuni* 81-176, which are both devoid of the Cj1321-Cj1325/6 locus. Furthermore, according to the results of whole-genome DNA microarray analysis, *C. jejuni* 81-176 falls into the non-livestock-associated clade (10). In *C. coli* VC167 and *C. jejuni* 81-176, flagellin glycosylation has been shown previously to be important in virulence, affecting microcolony formation, autoagglutination, and colonization in vivo, by using the ferret diarrheal disease model (21).

Scrutiny of *Campylobacter* genome sequence data revealed orthologues of the full locus (Cj1321 to Cj1325/6) in two further strains, *C. jejuni* subsp. *jejuni* 84-25 and CF93-6. In addition, orthologues of Cj1324 and Cj1325/6 were found in the closely related species *C. jejuni* subsp. *doylei* 269.27, *C. lari* RM2100, *C. upsaliensis* RM3195, and as previously reported, *C. coli* VC167 (36) but were absent in the whole-genome shotgun sequence of *C. coli* RM2228. Why some strains have all of the genes Cj1321 to Cj1325/6 and others do not is currently unknown, but this pattern may relate to the host specificities of different *Campylobacter* species. *C. jejuni* is highly recombinogenic, with multiple mechanisms to vary its glycan content, as exemplified by both the capsule and LOS loci (16, 27). The stability of the Cj1321-Cj1325/6 locus during laboratory passage was monitored, and the locus remained stable, indicating that it is not easily lost through recombination (S. L. Howard and B. W. Wren, unpublished data).

Previously, genes within the O-linked flagellum glycosylation locus have been shown to be essential for flagellation and motility in *C. jejuni* and *C. coli* (19). Mutagenesis in *pseB*, involved in the biosynthesis of pseudaminic acid, in strain *C. jejuni* 81-176 results in a strain with a nonmotile phenotype lacking the flagellar filament (19). Motility is essential for the successful colonization of the intestine in both animals and humans (23, 40, 41, 60). Gene-specific disruption of Cj1321, Cj1322, Cj1323, Cj1324, or Cj1325/6 or the removal of the entire locus (Cj1321 to Cj1325/6) did not affect motility. This finding supports recently published data from Reid et al., who reported that the motility of a Cj1324 transposon mutant was unaffected compared to that of the wild type (45), and data from previous studies of genes involved in the *ptm* pathway, which have shown that mutagenesis in Cj1332 (*ptmA*) and Cj1331 (*ptmB*) does affect motility (20, 62). The presence of flagella facilitated a top-down MS analysis of selected mutants

to determine whether genes in the Cj1321-Cj1325/6 locus encode novel glycan modifications to flagellin in strain 11168H.

The deletion of Cj1324 and the whole glycosylation locus (Cj1321 to Cj1325/6) resulted in significant reductions in autoagglutination after 24 h (to 54 and 60%, respectively, compared to 90% for 11168H). In addition, the ΔCj1324 and Δ(Cj1321-Cj1325/6) mutants showed significant decreases in hydrophobicity. The results of autoagglutination and hydrophobicity assays together with MS data demonstrate that, when Cj1324 is deleted, Leg5Am7Ac and Leg5AmNMe7Ac glycan modifications are absent, resulting in a change of the cell surface charge for the flagellum and the loss of aggregation. These phenotypic characteristics of the Cj1324 mutant appear to be due specifically to the alteration of the flagellin-associated glycan rather than alterations of other *C. jejuni* glycans such as LOS and the capsule. The LOS in the mutant appeared to be identical to that in the wild-type strain upon silver staining, the capsule appeared to be unaltered upon Alcian blue staining, and also the Penner serotyping result remained type 2 (unpublished data). The autoagglutination of *C. jejuni* 81-176 and *C. coli* VC167 is known to be associated with both the hydrophobicity of the bacteria and the presence of flagella (39). The loss of glycosylation with either PseAc or PseAm results in the failure of the strains to autoagglutinate (21). Thus, it has been proposed previously that interactions between these two glycans may both strengthen the flagellum and aid in autoaggregation through interactions between flagella (21). Here, we show that in 11168H, glycans Leg5Am7Ac and Leg5AmNMe7Ac also appear to have a contributory role in autoagglutination. No significant differences from the wild type were observed when the ΔCj1321 and ΔCj1325/6 mutants were analyzed in autoagglutination assays or when the ΔCj1321, ΔCj1322, ΔCj1323, and ΔCj1325/6 mutants were evaluated in hydrophobicity assays and chicken colonization studies. The precise roles of Cj1321, Cj1322, Cj1323, and Cj1325/6 remain to be determined. Additionally, properties such as autoagglutination and biofilm formation are multifactorial, so the presence of Cj1324 does not fully explain the mechanisms of these phenomena. Indeed, several of the non-livestock-associated strains that lack the Cj1321-Cj1325/6 locus, including strain 81-176, were still able to autoagglutinate and form biofilms (unpublished data), confirming that numerous factors are involved in these complex physiological processes. Adhesion and invasion assays using CKC and HEp2 and HD11 cells showed no significant differences between wild-type and mutant strains. In a recent study by Guerry et al. in which a *pseA* mutant of *C. jejuni* strain 81-176 (which can no longer synthesize the acetamidino form of pseudaminic acid) was tested for adherence to and invasion of INT407 cells, only minor reductions compared to those by the wild type were seen (21).

Reeser et al. reported that a *flaAB* mutation in *C. jejuni* M129 results in reduced biofilm formation (this mutant lacks flagellin and therefore glycan moieties are not present on the cell surface) (44). In our study, we have shown that the mutation of Cj1324 or the deletion of the whole locus of Cj1321 to Cj1325/6 in 11168H results in a significant reduction in biofilm formation after 7 days. Hydrophobic surface components of bacterial cells, such as flagella, are thought to help overcome repulsive forces, allowing the bacteria to attach to both hydrophobic and hydrophilic surfaces (14, 25). In addition, biofilm formation has been shown to be important in the colonization of the urinary tract by uropathogenic strains of *E. coli*, allowing the infection to persist through evasion of the host's defenses (1). Given that motility was unaffected in all of the mutants in this study, our results suggest that rather than the presence of flagella alone, Leg5Am7Ac and Leg5AmNMe7Ac modifications may contribute to the formation and/or the development of biofilms.

We investigated the role of O-linked glycosylation in colonization and persistence in the avian intestinal tract. In this study, Δ(Cj1321-Cj1325/6) and ΔCj1324 mutants exhibited prolonged low-level persistence in chickens compared to that of the parent strain, *C. jejuni* 11168H. Δ(Cj1321-Cj1325/6) complementation restored wild-type levels of colonization, and there was marginal restoration by ΔCj1324 complementation. The reasons for the lack of full restoration of persistence of the ΔCj1324 complemented strain are unclear. However, the counts of bacteria of both complemented strains in the avian intestinal tract were restored to the levels of the parent strain. These data suggest that the presence of CMP-Leg5Am7Ac and CMP-Leg5AmNMe7Ac is a contributory factor for the efficient colonization of the cecum. The Δ(Cj1321-Cj1325/6) mutant also exhibited significant defects in the colonization of various parts of the avian intestinal tract compared to the parent strain at 6 weeks postinfection, whereas statistically significant reductions in levels of colonization by the ΔCj1324 mutant were observed only in the cecal contents and in association with cecal tonsils. The reasons for the different counts of these mutant bacteria in distinct sites are unknown. The influences affecting the colonization of avian and human hosts by enteric bacteria such as *C. jejuni* are multifactorial and complex. These factors may involve autoagglutination, microcolony formation, surface charge-charge interactions, biofilm formation, survival under stress (e.g., low-pH stress), the ability to counteract host immune responses, and direct receptor-ligand interactions. *Campylobacter* species have a wide host range and are found in the gastrointestinal tracts and feces of many birds and mammals (47, 64). There is overlap in the host ranges of *C. jejuni* and *C. coli*, which both colonize chicken, pigs, and cattle, and it has been shown previously that genetic exchange between these two *Campylobacter* species occurs (12, 53). However, *C. jejuni* and *C. coli* also display host specificity, as they predominate in wild birds and pigs, respectively. We have shown that glycan modifications to flagellin of *C. jejuni* 11168H directly affect the colonization of chickens, and we propose that the modification of flagellin glycosylation may also play a role in the host specificity of *Campylobacter* colonization. Interestingly, strain 81-176, a non-livestock-associated strain originally isolated from milk, colonizes and persists at a lower level in chickens than 11168H (23).

HILIC-MS and precursor ion scanning were used to selectively analyze the metabolome of *C. jejuni* 11168H for biosynthetic sugar nucleotides. The metabolome of the ΔCj1324 mutant revealed the disappearance of CMP-Leg5Am7Ac, CMP-Leg5AmNMe7Ac, and CMP-Pse5Ac7Am. Both CMP-Leg5Am7Ac and CMP-Leg5AmNMe7Ac were restored upon complementation; however, CMP-Pse5Ac7Am was not detected, which may be a consequence of increased utilization of this nucleotide sugar through incorporation either as a flagellar glycan or as a substrate in the production of the *m/z* 711

CMP-2,3-di-*O*-methylglyceric acid PseAm derivative. CMP-Leg5Am7Ac and CMP-Leg5AmNMe7Ac were also detected in three strains of *C. jejuni* (11856, 12450, and 12567) which were classified as livestock associated by comparative phylogenomics and possessed Cj1324 (10). Furthermore, confirmation that Cj1324 has a role in the biosynthesis of these novel legionaminic acid derivatives was demonstrated through the analysis of four strains from the non-livestock-associated clade. 12241, 15168, 31467, and 43983 lack Cj1324, and HILIC-MS analysis revealed that CMP-Leg5Am7Ac and CMP-LegAmN Me7Ac were absent. Thus, we have shown by metabolomic analyses of additional *C. jejuni* strains that Cj1324 is involved in CMP-Leg5Am7Ac and CMP-LegAm7Ac synthesis and that it is not restricted to the sequenced strain 11168H. The variation of glycan modifications was exemplified by findings from the analysis of *C. jejuni* 80646, which in addition to lacking CMP-Leg5AmNMe7Ac lacks the two novel CMP-linked sugar intermediates (*m*/*z* 711 and 712). CMP-Leg5Am7Ac and CMP-Leg5AmNMe7Ac have also been found on the flagellin of *C. coli* VC167 (36). Although the precise biosynthetic pathway has yet to be determined, it has been proposed that in *C. coli*, *ptmG* and *ptmH* encode a CMP-Leg5Am7Ac acetamidinosynthase and an acetamidino-N-methyltransferase, respectively (36). We have shown that in *C. jejuni* 11168H, Cj1324 appears to have a function similar to that of *ptmG*, as the loss of Cj1324 results in a loss of metabolites similar to that in the equivalent mutant of *C. coli* VC167. The precise function of Cj1324 in the legionaminic acid biosynthetic pathway is currently being investigated through enzymatic analysis of recombinant Cj1324 protein.

It is estimated that between 40 and 80% of retail chickens are contaminated with *C. jejuni*. *C. jejuni* can colonize the small intestines of chickens at levels as high as 10^9 CFU per g of cecal contents without the birds' showing any ill effects (5). Combined with the low infectious dose for humans of ~500 *Campylobacter* organisms (6), this situation reinforces the importance of identifying strategies to reduce levels of colonization in poultry. This study has, for the first time, provided an explanation (the structural modification of the flagellin glycan) for why some *C. jejuni* strains are more likely than others to colonize chickens. The discovery of determinants such as Cj1324, which enhances the ability of *C. jejuni* to colonize chickens, should aid rational approaches to reduce the prevalence of this problematic pathogen in the food chain. This study has validated the utility of microarray-based methods for whole-genome comparisons using comparative phylogenomics in identifying host-specific determinants and confirms the important and diverse roles that glycosylated flagellins play in bacterial pathogens, even among members of the same species.

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