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Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence

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Summary

Analysis of the complete flagellin glycosylation locus of *Campylobacter jejuni* strain 81–176 revealed a less complex genomic organization than the corresponding region in the genome strain, *C. jejuni* NCTC 11168. Twenty-four of the 45 genes found between Cj1293 and Cj1337 in NCTC 11168 are missing in 81–176. Mutation of 6 new genes, in addition to three previously reported, resulted in a non-motile phenotype, consistent with a role in synthesis of pseudaminic acid (PseAc) or transfer of PseAc to flagellin. Mutation of Cj1316c or *pseA* had been shown to result in loss of the acetamidino form of pseudaminic acid (PseAm). Mutation of a second gene also resulted in loss of PseAm, as well as a minor modification that appears to be PseAm extended with *N*-acetyl-glutamic acid. Previously described mutants in *C. jejuni* 81–176 and *Campylobacter coli* VC167 that produced flagella lacking PseAm or PseAc failed to autoagglutinate. This suggests that interactions between modifications on adjacent flagella filaments are required for autoagglutination. 81–176 mutants defective in autoagglutination showed a modest reduction in adherence and invasion of INT407 cells. However, there was a qualitative difference in binding patterns to INT407 cells using GFP-labelled 81–176 and mutants lacking PseAm. A mutant lacking PseAm was attenuated in the ferret diarrheal disease model.

Introduction

Although originally considered a eukaryotic-specific phenomenon, there is an increased awareness of prokaryotic glycoproteins, particularly bacterial surface proteins (reviewed in Schmidt *et al*., 2003 and Szymanski and Wren, 2005). The most heavily glycosylated bacterial proteins described to date are the flagellins from *Campylobacter jejuni* and *Campylobacter coli*. Flagellin from *C. jejuni* strain 81–176 has been shown to be glycosylated at 19 serine or threonine residues, and *C. coli* VC167 at approximately 16 sites (Thibault *et al.* 2001; Logan *et al*., 2002). Although the glycans show variability among strains and can confer serospecificity (Logan *et al.*, 2002), the carbohydrate modifications appear to be based on 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid (pseudaminic acid), a 9-carbon sugar that is structurally similar to sialic acid (NeuNAc; Thibault *et al.*, 2001). The major modifications identified on flagellin from *C. jejuni* strain 81–176 were pseudaminic acid

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(PseAc, m/z 317) and an acetamidino form of pseudaminic acid (PseAm, m/z 316). There were also minor amounts of an acetylated form of pseudaminic acid (PseOAc, m/z 359), and a form in which the PseAm sugar had an *N*-acetyl glutamic acid attached (PseAmOGlnNAc; m/z 486; Schirm *et al.*, 2005).

All genes that have been shown to be involved in flagellin glycosylation to date map near the two flagellin structural genes, *flaA* and *flaB*, in a region that is one of the more variable regions in the *C. jejuni* chromosome (Dorrell *et al.*, 2001; Fouts *et al.*, 2005; Pearson *et al.*, 2004) and includes genes encoding enzymes that have recently been shown to be part of the PseAc biosynthetic pathway (Chou *et al.*, 2005; Schoenhofen *et al.*, 2006), as shown in Fig. 1. Six genes in this region in *C. jejuni* 81–176 have been mutated. A mutant in Cj1293 or *pseB*, which encodes the first step in biosynthesis of PseAc (Schoenhofen *et al.*, 2006), was non-motile and accumulated unglycosylated flagellin intracellularly, demonstrating a requirement for glycosylation of flagellin for flagella filament biogenesis (Goon *et al.*, 2003). Mutants in Cj1314c, Cj1315c, as well as Cj1317, which encodes the enzyme recently shown to condense a deoxyhexose precursor with PEP to produce pseudaminic acid (Chou *et al.*, 2005), were also non-motile (Thibault *et al.*, 2001). Karlyshev *et al.* 2002 have reported on a family of related genes in NCTC 11168 called *maf* genes (for *m*otility *a*ccessory *f*actor). Mutation of one of these *maf* genes in NCTC 11168 (*maf*5 or Cj1337) resulted in a non-motile phenotype, and more recently this same group has reported that the corresponding mutation in *C. jejuni* 81–176 also resulted in a non-motile phenotype (Karlyshev and Wren, 2005). The product of the fourth 81–176 gene, Cj1316c or *pseA*, appears to synthesize PseAm directly from PseAc by transfer of an acetamidino group (Thibault *et al.*, 2001). Thus, a mutant in this gene was fully motile, but flagellin from the mutant had all PseAm modifications replaced with PseAc (Thibault *et al.*, 2001). Other strains of campylobacter appear to have an additional, alternate pathway for synthesis of a related form of PseAm (Guerry *et al.*, 1996; Parkhill *et al.*, 2000; Logan *et al.*, 2002; Fouts *et al.*, 2005). Genetic analysis in *C. coli* VC167 has identified 6 genes, including additional homologs of NeuNAc biosynthetic genes, that are involved in direct synthesis of PseAm independently of PseAc synthesis, and these have been termed *ptmA-F* (Guerry *et al.*, 1996; Logan *et al.*, 2002). Thus, there appears to be variability in PseAm biosynthetic pathways among *Campylobacter* strains. Strains like NCTC 11168 have homologs of both the *ptm* genes and *pseA*, and would be predicted to have two distinct pathways directed towards the synthesis of PseAm. In contrast, 81–176 has a single pathway by which all PseAm is synthesized via a PseAc precursor, and *C. coli* VC167, which lacks a functional *pseA* gene, can synthesize PseAm only by the *ptm* pathway (Logan *et al.*, 2002).

Here we characterize the complete flagellin glycosylation locus of *C. jejuni* strain 81–176. The simplicity of this locus in strain 81–176 has facilitated identification of the minimum number of genes required for synthesis of the two major flagellin modifications, PseAc and PseAm. Also, the glycans on flagellin have been suggested to play a role in autoagglutination and virulence (Golden and Acheson, 2002), and we have reexamined this association using a collection of defined mutants in both 81–176 and *C. coli* VC167 (Guerry *et al.*, 1996; Thibault *et al.*, 2001; Logan *et al.*, 2002).

Results

DNA sequence analysis of the flagellin glycosylation locus of 81–176

A 32.4 kb contiguous region of the *C. jejuni* strain 81–176 chromosome that spanned Cj1291 to Cj1342 was sequenced, as shown in Table 1. Strain 81–176 is missing 25 genes that are found in NCTC 11168, namely Cj1296, Cj1297, Cj1301, Cj1304, Cj1306-Cj1310, Cj1318-Cj1332, and Cj1340. There are some genes that appear rearranged in 81–176 compared to the NCTC 11168 chromosome. Thus, the homolog of Cj1300c maps between Cj1295 and Cj1298 in 81–176, in place of Cj1296 and Cj1297. Also, the 81–176 gene that shows the highest

homology to Cj1333 maps between Cj1334 and Cj1337. Of the seven *maf* gene homologs described by Karlyshev *et al.* 2002 in NCTC 11168, only five, Cj1333 (*maf2*), Cj1334 (*maf3*), Cj1337 (*maf5*), Cj1341c (*maf6*) and Cj1342c (*maf7*), were found in 81–176.

There was one gene unique to 81–176 that showed no homology to other known campylobacter genes (Fouts *et al.*, 2005; Parkhill *et al.*, 2000). This gene, termed CjB1301, encodes a predicted protein of 52.2 kDa, pI 9.4. The predicted protein showed significant homology to a dehydratase/ketoreductase domain of BryA, an enzyme from a bacterial symbiont of a marine bryozoan that is involved in polyketide synthesis (Hildebrand *et al.*, 2004).

There were three genes in this region that had homopolymeric tracts of 9 C's or G's within the coding region, suggesting possible slip strand mismatch repair; these were Cj1295, Cj1305c and Cj1342c. However, Cj1295 was truncated as cloned from 81–176 into two ORFs by what appears to be slip strand mismatch repair at a homopolymeric tract of As. However, none of these three genes appear to be involved in flagellin glycosylation (see below).

Mutational analysis of the flagellin glycosylation locus

As mentioned above, mutants in 6 genes in this locus (Cj1293/*pseB*, Cj1317, Cj1316c/*pseA*, Cj1315c, Cj1314c and Cj1337) in 81–176 have been reported previously (Thibault *et al.*, 2001; Goon *et al.*, 2003; Karlyshev and Wren, 2005). A total of 17 additional genes in this region were mutated. The two genes that were not mutated were Cj1299 and Cj1292. No transposon insertion was isolated in Cj1299; in the case of Cj1292 a transposon insertion was obtained in *E. coli*, but multiple attempts to electroporate the mutated allele into 81–176 were unsuccessful. All mutants were screened for motility and, if positive, flagellins were purified and characterized on IEF gels (Doig *et al.*1995; Thibault *et al*., 2001; Logan *et al.*, 2002; Goon *et al*., 2003). Mutants displaying an altered IEF pattern were then examined by mass spectrometry. The data are summarized in Table 1 and those genes that appear to be involved in glycosylation have been designated "*pse*" (for biosynthesis or attachment of PseAc or PseAm; Thibault *et al*., 2001; Goon *et al*., 2003) as indicated in Table 1, and this nomenclature will be used throughout.

In addition to the previously described non-motile 81–176 mutants in Cj1317 or *pseI* (Thibault *et al.*, 2001) and Cj1337 or *pseE* (Karlyshev and Wren, 2005), mutation of 3 additional genes resulted in a totally non-motile phenotype. These included the homolog of Cj1311, which encodes a protein with similarity to NeuA, the enzyme that activates NeuNAc; this gene has been reannotated as *pseF*. Mutation of *pseF* has been shown to result in a non-motile phenotype in *C. jejuni* strain G1, but not NCTC 11168 (Linton *et al.*, 2000), which is likely a reflection of the presence of the alternate *ptm* pathway for PseAm synthesis in the latter strain (Parkhill *et al.*, 2000). 81–176 mutants in Cj1312 and Cj1313, which were originally annotated in the NCTC 11168 genome as a putative flagellar genes based on homology to putative flagellin glycosylation genes of *Caulobacter crescentus*, were also non-motile; these genes have been called *pseG* and *pseH*, respectively. Mutation of Cj1294, designated *pseC* (Schoenhofen *et al*., 2006), also resulted in a non-motile phenotype, and it has been shown to be involved in synthesis of PseAc (Fig. 1; Schoenhofen *et al*., 2006). An 81–176 mutant in Cj1337 generated in this study was also non-motile, as previously reported for NCTC 11168 *maf*5 (Karlyshev *et al.*, 2002) and more recently for 81–176 (Karlyshev and Wren, 2005); this 81–176 gene has been called *pseE*. Mutants in *pseF*, *pseG*, *pseH* and *pseE* lacked flagella filaments and hook structures as determined by transmission electron microscopy (data not shown). When the *pseC* and *pseE* mutants were complemented *in trans* using an expression plasmid (Larsen *et al.*, 2004), motility was restored (data not shown). Although the mutations in *pseG* and *pseH* were not complemented, 20 independent mutations in *pseG* and 14 independent mutations in *pseH* were non-motile, suggesting that the phenotype was not due to phase variation at a distal locus.

In addition to the previously reported Cj1314c and Cj1315c (Thibault *et al.*, 2001), mutation of 11 other genes resulted in a fully motile phenotype with no discernible changes in flagellin glycosylation based on IEF analysis. These genes, most of which encode proteins of unknown function, were Cj1295, Cj1300c, Cj1298, CjB1301, Cj1302, Cj1303, Cj1304, Cj1305c, Cj1334, Cj1341, and Cj1342. Mutation of Cj1333 in 81–176 is discussed below.

Characterization of a mutant in Cj1333

Although Cj1333 was designated as *maf2* based on homology to other *maf* genes (Karlyshev et al, 2002), a mutant in 81–176 was fully motile. IEF analysis of the flagellin from a Cj1333 mutant was intermediate between wildtype and a *pseA* mutant as shown in Fig. 2A. The molecular mass profile obtained from the nanoelectrospray mass spectrometry analysis of *C. jejuni* Cj1333 mutant flagellin is presented in Figure 3A, and shows a distinct pattern to that of the wild type strain of *C. jejuni* or the Cj1316c mutant (Thibault *et al.*, 2001). The MS-MS analysis of the multiply-charged flagellin precursor ions at m/z 985.5 revealed the presence of PseAc residues (m/z 317) with no detectable PseAm (Figure 3B). The modified residue previously observed in 81–176 flagellin at m/z 486 is now observed at m/z 487 suggesting a conversion of PseAm to PseAc. Confirmation of this was obtained from the second generation product ion of m/z 487 produced from in-source fragmentation of the flagellin multiply-charged ions (Figure 3C). The MS-MS spectrum of m/z 487 clearly shows a fragment ion at m/z 171 for the acylium ion of the GlnAc residue together with fragment ions at m/z 317, 299 and 281 corresponding to the oxonium ion of PseAc and consecutive H_2O losses. This top-down analysis indicated that Cj1333 mutant strain has lost the ability to decorate flagellin with PseAm (m/z 316) and PseAmOGlnAc (m/z 486) residues and can now only glycosylate the flagellin monomer with *N*-acetylated monosaccharide residues (PseAc, m/z 317) which at certain sites are further substituted with OGlnAc (m/z 487). Complementation of the mutant *in trans* on a shuttle plasmid resulted in a restoration of the wildtype IEF pattern, as shown in Fig. 2A. Intact mass analysis of purified flagellin from the complemented mutant showed a return to the more heterogeneous wildtype profile (Fig. 4A). The MS/MS fragmentation pattern of the precursor ion m/z 486 from the complemented mutant (Fig. 4B) is consistent with that obtained from the precursor ion m/z 486 from 81–176 (see Schirm et al., 2005). These data confirmed restoration of both the m/z 316 and 486 groups on flagellin, although the amounts of these glycans were reduced compared to wildtype, suggesting possible instability of the complementing plasmid. The data suggest that the product of Cj1333 is involved in attachment of PseAm and the gene has been annotated as *pseD*.

Changes in flagellin modifications affect autoagglutination

Autoagglutination (AAG) of *C. jejuni* has been shown to be associated with flagellin (Misawa and Blaser, 2000), and possibly flagellin glycans (Golden and Acheson, 2002), and to be dependent on quorum sensing (Jeon *et al.*, 2003). We examined this further using a set of mutants described in Table 2. As shown in Fig. 5A, a mutant of 81–176 in *flaA* (Yao *et al.*, 1994) failed to autoagglutinate (column 2), similar to results reported by Misawa and Blaser (2000) for a *flaA flaB* mutant of 81–176. A *luxS* mutant of another *C. jejuni* strain has been reported to be reduced in both AAG and motility (Jeon *et al.*, 2003). However, an 81–176 *luxS* mutant was fully motile and flagellin purified from the strain showed no change in IEF pattern (data not shown). The *luxS* mutant (column 6) showed an intermediate level of AAG between the levels of *flaA* (column 2) and wildtype 81–176 (column 1). A paralyzed mutant of 81–176 that synthesizes a filament, but is non-motile (Yao *et al*., 1994) autoagglutinated as well as wildtype (column 3), indicating that flagella structure rather than motility is required for AAG. We also examined previously described mutants of 81–176 in two other surface structures, *kpsM*, lacking capsular polysaccharide (Bacon *et al.*, 2001), and *neuC*, which has an altered LOS core lacking NeuNAc (Guerry *et al.*, 2001). The *neuC* mutant (column 5)

showed an AAG level comparable to *luxS*, whereas the capsule mutant was only slightly less proficient in AGG (column 4) than wildtype.

Fig. 5B shows AGG using 81–176 glycosylation mutants. Mutants in *pseA* and *pseD* both lacked PseAm on flagellin and both failed to autoagglutinate (columns 2 and 4). AAG was restored when the mutations were complemented either *in trans* for *pseD* (column 3) or by insertion of a wildtype allele into the arylsulfatase (*astA*) gene for *pseA* (column 5). A control for the *atsA* insertion is also shown (*atsA::aph3*; column 6). The mutant in CjB1301 (column 7), which showed no detectable flagellin changes by IEF (Table 1), agglutinated as well as wildtype (column 1).

VC167 has the genes encoding the *ptm* pathway for synthesis of PseAm independent of PseAc synthesis, and the *pseA* gene, which converts PseAc to PseAm in *C. jejuni* 81–176 (Goon *et al.*, 2003), is a pseudogene in VC167 (Logan *et al.*, 2002). Thus, mutants have been described in *C. coli* VC167 that lack either all PseAm or lack all PseAc (Guerry *et al.*, 1996; Logan *et al.*, 2002). We used these mutants in AAG assays to confirm and extend the observations made with *C. jejuni* 81–176 and the results are shown in Fig. 5C. The VC167 *ptmD* mutant, that has flagellin substituted with PseAc in place of PseAm, failed to autoagglutinate (column 2), but AAG was restored by complementation with the *ptmD* gene *in trans* (column 3). A mutant in *pseA*, which has PseAm moieties in sites normally occupied by PseAc (Goon *et al.*, 2003), also failed to autoagglutinate (column 4); the ability of this mutant to autoagglutinate could also be complemented *in trans* (column 5).

Effect of flagellin modification/autoagglutination on adherence and invasion of intestinal epithelial cells

Since the ability of *C. jejuni* to autoagglutinate has been associated with changes in adherence and/or invasion (Misawa and Blaser, 2000; Golden and Acheson, 2002), the ability of wildtype 81–176 and the *pseA* mutant to adhere to and invade intestinal epithelial cells was compared. The results, shown in Fig 6A, indicated that the *pseA* mutant (column 2) adhered to INT407 cells at about 20% the level of wildtype (column 1) and that the defect was restored in the complemented strain (column 3). A control, in which only a kanamycin resistance gene was inserted into *atsA* adhered at levels comparable to wildtype (column 4). The effect of mutation of *pseA* on invasion of INT407 cells was more modest (Fig. 6B); the *pseA* mutant (column 2) invaded at about 42% the level of wildtype (column 1), although invasion levels were increased in the complemented strain (column 3).

Comparison of patterns of adherence of GFP tagged bacteria to INT407 cells

Since autoagglutination is often associated with microcolony formation in other pathogens (Skurnik *et al.*, 1984; Nataro *et al.*, 1987; Blake *et al.*, 1995; Chiang *et al.*, 1995; Roggenkamp *et al.*, 1995; Bieber *et al.*, 1998; Knutton *et al.*, 1999; Frick *et al.*, 2000; Kirn *et al.*, 2000; Schembri *et al.*, 2001; Sherlock *et al.*, 2005), the patterns of adherence of wildtype 81–176 and the *pseA* mutant, each of which was carrying pCE111/28/GFP (Hickey *et al.*, 2005), to INT407 cells were compared. Examination of INT407 cells infected with either strain at 6 h showed single fluorescent bacteria (data not shown). However, after 18 h of incubation, distinct patterns could be observed in the wildtype 81–176 but not the *pseA* mutant (Fig. 7A). The wildtype strain often formed apparent chains, which were also visible in the phase contrast images, while the mutant adhered as single cells. By 24 h (Fig. 7B and 7C), the wildtype strain had formed apparent microcolonies and chains of bacteria were often visible protruding from the microcolony. The amount of binding of the *pseA* mutant at 24 h appeared to increase, but the pattern of mutant binding remained more diffuse than that of wildtype (Fig. 7B). The pattern of binding of a GFP-tagged version of the *pseD* mutant was indistinguishable from that of the *pseA* mutant (data not shown).

Effect of loss of PseAm on virulence in vivo

To determine if these differences in adherence patterns corresponded to decreased virulence *in vivo*, the *pseA* mutant and the complemented strain were compared to wildtype 81–176 in the ferret diarrheal disease model. A total of 16 animals were fed 3.8–4.5 x 10^{10} CFU of each strain and the numbers of animals developing diarrhea over the next 4 days was monitored. The results are summarized in Fig. 8. A total of 10/16 animals fed wildtype 81–176 and the complemented mutant developed diarrhea compared to only 3/16 animals fed the *pseA* mutant (P=0.029).

Discussion

The flagellin glycosylation locus of *C. jejuni* 81–176 is much simpler than the corresponding locus in *C. jejuni* NCTC 11168, having approximately half the number of genes and lacking the so-called “second set” of sialic acid genes or *ptm* genes that are directly involved in synthesis of PseAm (Guerry *et al.*, 1996; Logan *et al.*, 2002). Thus, the region corresponding to the 45 genes spanning Cj1293 to Cj1337 in NCTC 11168 contains 21 genes in 81–176, and mutation of only 9 of these resulted in a phenotype affecting modification of flagellin with either PseAc or PseAm. Mutation of a total of seven genes resulted in a non-motile phenotype. This suggests that these genes are involved in either synthesis of PseAc, through which PseAm is synthesized in this strain, or attachment of both PseAc and PseAm to flagellin. As shown in Fig. 1, four of these genes, *pseB, pseC, pseF* and *pseI*, encode enzymes in the PseAc pathway. The roles of PseG, PseH, and PseE remain to be elucidated.

The loss of PseAm in mutants of *pseA* and *pseD* suggests that both genes encode proteins that are part of a pathway in biosynthesis of PseAm from PseAc or transfer of PseAm to flagellin. While flagellin from the *pseD* mutant had lost both PseAm and the novel group of m/z 486, this mutant now appeared capable of transfer of OGlnAc to PseAc (m/z 487). In contrast, the *pseA* mutant, while losing both m/z 316 and m/z 486 was unable to transfer OGlnAc to PseAc for reasons that remain to be determined. The enzyme or enzymes responsible for attachment of this novel OGlnAc group remain unknown, although the data suggest that PseA may influence this process. Since the predicted phenotype of a mutant lacking this m/z 486 group would be motile with no change in IEF pattern, there are numerous candidate genes within the glycosylation locus, although identification of this gene will require MS analysis of flagellins from each mutant.

All five *maf* gene homologs were mutated in 81–176 but mutants in only two, *pseD* and *pseE*, showed an observable change in flagellin. As reported here and previously by another group (Karlyshev and Wren, 2005), the 81–176 *pseE* mutant is non-motile, which is the predicted phenotype of *maf* mutants in NCTC 11168 (Karlyshev *et al.*, 2002). Colegio *et al.* 2001 reported that a transposon insertion into Cj1318, one of the *maf* genes, of 81–176 was non-motile, but the data presented here indicate that there is no copy of Cj1318 in the flagellin glycosylation locus of 81–176. Either there is a Cj1318 copy elsewhere on the chromosome, or the insertion was actually in *pseE*, which shows the highest homology (39% identity/53% similarity) to Cj1318. However, the motile phenotype and the observed changes in glycosylation of the *pseE* mutant are different than what is predicted for a *maf* gene phenotype.

Spontaneous autoagglutination of *C. jejuni* strains has long been recognized as a problem in serotyping schemes (Lior *et al.*, 1982; Penner and Hennessy, 1980; Wong *et al.*, 1985). The phenomenon was shown to be associated with flagella by Misawa and Blaser (2000) and shown in the same study to correlate with cell surface hydrophobicity. A *kpsM* mutant of 81–176 showed reduced hydrophobicity (Bacon *et al.*, 2001), a result that is consistent with a reduction in AAG for this mutant. The involvement of flagella in AAG was confirmed in a random transposon mutagenesis screen of *C. jejuni* strain 480 (Golden and Acheson, 2002). In that

study Golden and Acheson also found non-agglutinating mutants in genes in the glycosylation locus, although the genes were not further characterized and they first reported reduced AAG in *ptmA* and *ptmB* mutants of VC167. Here, we have demonstrated that all mutants in 81–176 and *C. coli* VC167 that result in loss of PseAm residues from flagellin fail to autoagglutinate. Since PseAm is made via PseAc in 81–176, it is not possible to construct an 81–176 mutant that can decorate flagellin with only PseAm. However, we have previously described such a mutant in *C. coli* VC167 defective in *pseB* (Goon *et al.*, 2003), and that mutant also showed reduced AAG. Flagellin from the VC167 mutant in *pseB* also had an unusual phenotype in that it was more sensitive to dissociation in 1% SDS, which is used during the flagellin purification scheme (Goon *et al.*, 2003), and suggests that interactions among residues of PseAc and PseAm on flagellin monomers within the filament enhances the physical strength of the flagellum. The requirement for both PseAm and PseAc in the AAG process also suggests that interactions of residues across flagella filaments may be critical for the aggregative interaction. The slight reduction in AAG in a NeuNAc mutant was unexpected and requires additional investigation, but it may be that there are heterophilic interactions between PseAm residues on flagellin and the structurally related NeuNAc on LOS, similar to interactions seen between opacity proteins and sialylated LOS in *Neisseria* (Blake *et al.*, 1995). This is the first report, to our knowledge, of the role of a glycan on a glycoprotein in bacterial autoagglutination. Although the glycoprotein adhesin TibA from enterotoxigenic *E. coli* mediates autoagglutination, the glycan is required for attachment to epithelial cells, but not for agglutination (Benz and Schmidt, 2001; Sherlock *et al.*, 2005).

AAG has been demonstrated to be critical for virulence for a variety of pathogens, and can play a role in adherence, microcolony formation, biofilm formation, and resistance to acid and phagocytosis (Skurnik *et al.*, 1984; Galdiero *et al.*, 1988; Chiang *et al.*, 1995; Frick *et al.*, 2000; Sherlock *et al.*, 2005). In two previous studies on AAG of *C. jejuni* (Misawa and Blaser, 2000; Golden and Acheson, 2002), there appeared to be an association with adherence or invasion of intestinal epithelial cells. The quantitative defects in adherence and invasion of intestinal epithelial cells of the *pseA* mutant of 81–176 in a standard 2 hour assay were relatively minor. However, wildtype 81–176 appears able to form distinct microcolonies on INT407 cells upon longer incubation, while mutants lacking PseAm cannot. This distinction *in vitro* correlates well with the observed attenuation of the *pseA* mutant *in vivo* in the ferret model. This suggests that the changes in glycosylation/AAG relate more to microcolony formation during intestinal colonization rather than to primary adherence, a distinction that has been made for several other pathogens (Bieber *et al.*, 1998; Blake *et al.*, 1995; Chiang *et al.*, 1995; Frick *et al.*, 2000; Kirn *et al.*, 2000; Kirn and Taylor, 2005; Knutton *et al.*, 1999). The precise role of flagellin glycosylation in the biology and pathogenesis of *C. jejuni* will require additional investigation, but the data presented here suggest that the glycans decorating the surface of the flagella filament play a key role in virulence.

Experimental Procedures

Strains and growth conditions

C. jejuni 81–176 has been described (Black *et al.*, 1988). *Campylobacter* strains were grown routinely in Mueller Hinton agar under microaerobic conditions. Media were supplemented with chloramphenicol (10 μg/ml), trimethoprim (10 μg/ml) or kanamycin (25 μg/ml) when appropriate. *Escherichia coli* XL-1 Blue was the host for λZAP Express cloning experiments and DH5α was the host for subcloning and routine DNA manipulation. Shuttle plasmids were mobilized from *E. coli* DH5α using RK212.2 into *C. jejuni*, as previously described (Guerry *et al.*, 1994). Wildtype 81–176 and mutants were tagged with GFP by conjugal transfer of shuttle plasmid pCPE111/28/GFP (Hickey *et al.*, 2005).

DNA cloning and generation of mutants

Genes were identified from partial *Sau*3A libraries of *C. jejuni* 81–176 constructed in λZAP Express (Stratagene, La Jolla, CA). Cloned DNAs were subjected to sequence analyses using an Applied Biosystems Model 3100 DNA sequencer. Custom sequencing primers were synthesized on a Perkin Elmer Applied Biosystems Model 294 DNA synthesizer. Additional DNA sequence was obtained following transposition into some clones using an *in vitro* Tn5-based tranposition system (Epicentre, Madison, WI) with a campylobacter chloramphenicol resistance (*cat*) cassette and primers within the cassette, as previously described (Guerry *et al.*, 2000). Gaps in the region were closed by polymerase chain reaction (PCR) using the proof-reading polymerase *Pfu*I (Stratagene, LaJolla, CA). Transposon insertions identified during the sequencing phase were used to generate knockout mutations in *C. jejuni* 81–176 by electroporation. All mutants were confirmed to have undergone a double crossover by PCR with primers that bracketed the insertion point of the transposon. The position of transposon insertions in genes in the flagellin glycosylation locus are listed in Table 1. A non-polar transposon insertion that mapped 37 bp from the translational start of *luxS* was used to generate a *luxS::cat* mutant in *C. jejuni* 81–176.

Purification of flagellin

Flagellins were purified as described previously (Power *et al.* 1994).

Complementation of mutants in trans

Three mutants were complemented by expressing the wildtype alleles in a kanamycin resistant σ^{28} expression vector, pRY107/28 (Larsen *et al.*, 2004). Each final construction was mobilized from *E. coli* DH5α into the respective mutant by RK212.1 with selection on trimethoprim, kanamycin and chloramphenicol (Labigne-Roussel *et al.*, 1987). The *pseD* gene was PCR amplified with *Pfu*I high fidelity polymerase (Stratagene) using the following primers: Cj1333F: 5’-CGGGATCCATGAAATTTAATTTAAATCAAAAAGAGC-3’ and Cj1333R: 5’-CCGCTCGAGTTCATTTGTTTGCATTTTTTATCC-3’. The primers introduced *Bam*HI and *Xho*I sites, respectively. Following digestion with these restriction enzymes (New England Biolabs, Beverly, MA) the 1.9 bp amplicon was cloned into pCE107/28 (Larsen *et al.*, 2004) that had also been cut with *Bam*HI and *Xho*I.

The *pseE* gene was PCR amplified using Advantage HF2 high fidelity polymerase (BD Sciences, Clontech, San Jose, CA) with the following primers that introduced *Bam*HI and *Eco*R1 sites: Cj1337F: 5’-CGGGATCCCGATGCAAACAAATGAAATTTTTAAAAAAAATTTAG-3’ and Cj1337R: 5’-GGAATTCCTTAGATTAAGCTTCTTTTTTCTAGCTCATCC-3’. The resulting 1.9 kb amplicon was digested with *Bam*HI and *Eco*R1 and cloned into pCE107/28 (Larsen et al, 2004).

The *pseC* gene was PCR amplified using Advantage HF2 high fidelity polymerase with the following primers that introduced *Bam*HI and *Eco*R1 sites, respectively: Cj1294F: 5’-CGGGATCCCGATGATTACTTATTCTCATCAAAATAATGATC-3’ and Cj1294R: 5’-GGAATTCCTTATCCACAATATCCCTTTTTAACTTTTTC-3’. The resulting 1131 bp product was digested with *Bam*HI and *Eco*R1 and cloned into pCE107/28.

Complementation of Cj1316c by insertion into atsA

Although *trans* complementation of *pseA* has been described previously (Thibault *et al.*, 2001), a strain was constructed in which a wildtype allele of *pseA* was inserted into the arylsulfatase gene (*atsA*; Yao and Guerry, 1996) of the 81–176 *pseA* mutant. This was done because some plasmid constructions are lost at relatively high frequency without antibiotic

selection during adherence and invasion assays (Guerry, unpublished observations). The *pseA* gene was amplified by PCR from 81–176 using the following primers: 5'-TGTATGGAATAATCCTTAGGTGGTG-3' and 5'-AGTTTTACCCGCTTTGCTTGC-3'. The PCR reaction was done for 30 cycles of 95C/1 min; 52C/1 min; 72C/1min using *Pfu*I, and the 517 bp product was cloned into PCR-Script (Stratagene, La Jolla, CA). Clones were sequenced with the appropriate forward and reverse sequencing primers to determine the orientation of the *pseA* gene and to verify the sequence of the PCR amplicon. A clone containing *pseA* oriented such that the 3' end was adjacent to the *Pst*I site was selected for further manipulation. A kanamycin resistance cassette (*aph3*) from pILL600 (Labigne-Roussel *et al.*, 1987) was subsequently cloned into the *Pst*I site of this plasmid. The orientation of this cassette was determined to be in the same orientation as the *pseA* gene by sequence analysis. A *Not*I-*Xho*I fragment containing both *pseA* and Km^r was purified, made blunt-ended with Klenow I, and cloned into the unique *Eco*RV site within the *atsA* gene on pYG660 (Yao and Guerry, 1996). This plasmid was used to electroporate 81–176 *pseA::cat* (Thibault et al, 2001) to kanamycin resistance. Km^r colonies were screened for loss of arylsulfatase activity by growth on MH agar containing a chromogenic substrate (X-S; Sigma), as previously described (Yao and Guerry, 1996). Km^r Colonies that were phenotypically Ats-were confirmed to have the predicted genotype by PCR. One such mutant was further characterized. A control strain was also generated in which the Km^r cassette was inserted into *atsA* of 81–176.

Motility testing

Motility of mutants was compared with that of wildtype on semi-solid (0.4%) MH agar plates as previously described (Guerry *et al.*, 1991).

IEF gels

Isoelectric focusing gels were prepared as previously described (Doig *et al.*, 1995; Goon *et al.*, 2003; Logan *et al.*, 2002; Thibault *et al.*, 2001).

Mass spectroscopy

Flagellin extracts were dialyzed in water (0.2% formic acid, FA) using Centricon YM-30 membrane filters (Millipore) to a final concentration of 0.2 mg/ml prior to intact mass analyses on a Waters Q-TOF™ Ultima mass spectrometer. This solution was infused into the mass spectrometer at a flow rate of 0.5 ul/min. External calibration of the TOF was performed using a 150 fmoles/ml solution of Glu-Fibrinopeptide B (50% aqueous methanol, 0.2% formic acid) and provided mass accuracy typically within ± 0.07 m/z unit across the acquisition range (m/=50–1400). Molecular mass profiles were obtained through spectal deconvolution using MaxEnt (MassLynz softwar, Waters). Collision-induced dissocation (CID) experiments were performed using argon as collision gas with differential voltage values of 10–25 V between the collision cell and the incoming ions. Second generation fragment ion spectra were obtained by increasing the RF lens 1 voltage from 50 to 125 V, thereby forming fragment ions in the high-pressure region of the skimmer/cone region of the mass spectrometer.

Autoagglutination assays

AAG assays were done as described by Misawa and Blaser (2000). Basically, bacterial growth from a MH plate was resuspended in PBS, pH 7.4 to an OD_{600} 1.0. Two ml of each suspension was transferred to 12 x 75 mm polypropylene tubes, and incubated at room temperature for 24 h. One ml from the top of each tube was carefully removed and the OD_{600} measured. The drop in OD_{600} from the initial setting of 1.0 reflected the degree of autoagglutination.

Adherence and invasion assays

Adherence and invasion assays to INT407 cells were done at an MOI of approximately 20, as previously described (Oelschlaeger *et al.*, 1993; Yao *et al.*, 1997). For adherence assays, bacteria were incubated with the monolayer for 2 h at 37°C in 5% CO_2/95% air. The cells were then washed four times with Hank's balanced salt solution with strong agitation for 2 min prior to lysing the monolayer with 0.01% Triton X-100 and enumeration of total bacteria by plate count. For determination of invasion, the monolayer was washed twice with Hank's balanced salt solution after the 2 h incubation, and fresh, pre-warmed medium containing gentamicin at 100 ug/ml was added to kill extracellular bacteria. After 2 h incubation, the monolayer was washed twice with Hank's balanced salt solution, and lysed with 0.01% Triton X-100. The released intracellular bacteria were enumerated by plate count. For adherence assays using GFP labeled bacteria, INT407 cells were seeded onto cover slips. After infection with GFP labeled bacteria, the assays were incubated at 37°C for 6, 18 and 24 h and washed four times with Hank's Balanced Salt Solution prior to examination in a Nikon Eclipse E400 fluorescence microscope. Images were photographed with an Olympus camera using MagnaFire software and enhanced with Adobe Photoshop 7.0.

Electron microscopy

Mutants were examined by transmission electron microscopy following negative staining with uranyl acetate to determine the presence or absence of a flagella filament.

Ferret diarrheal model

Campylobacter-free, six-week old female ferrets were purchased from Triple F Farms (Sayre, PA). After one week acclimation, animals were infected with 1.4–2.7 x 10^{10} bacteria by oral gavage as previously described (1) and observed for signs of diarrheal illness for four days. Experiments were done in duplicate with eight animals per group or a total of 16 animals per strain. The experiments were conducted in compliance with the Animal Welfare Act and according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. The *P* value was calculated by Fishers Exact Test using GraphPad Instat software (GraphPad, San Diego, CA).

Accession number

The complete flagellin glycosylation locus of *C. jejuni* 81–176 has been deposited in Genbank as AY102622.

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References

Bacon DJ, Szymanski CM, Burr DH, RP Silver RP, Alm RA, Guerry P. A phase variable capsule is involved in virulence of *Campylobacter jejuni* 81–176. Mol Microbiol 2001;40:769–777. [PubMed: 11359581]

Benz I, Schmidt MA. Glycoylation with heptose residues mediated by the *aah* gene product is essential for adherence of the AIDA adhesin. Mol Microbiol 2001;40:1403–1413. [PubMed: 11442838]

Bieber D, Ramer SW, Wu CY, Murray WJ, Tobe T, Fernandez R, Schoolnik GK. Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli.* Science 1998;280:2114–2118. [PubMed: 9641917]

Black RE, Levine MM, Clements ML, Hughes TP, Blaser MJ. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis 1988;157:472–479. [PubMed: 3343522]

Blake MS, Blake CM, Apicella MA, Mandrell RE. Gonococcal opacity: lectin-like interactions between Opa proteins and lipooligosaccharide. Infect Immun 1995;63:1434–1439. [PubMed: 7890406]

Chiang SL, Taylor RK, Koomey M, Mekalanos JJ. Single amino acid substitutions in the N-terminus of *Vibrio cholerae* TcpA affect colonization, autoagglutination and serum resistance. Mol Microbiol 1995;17:1131–1142.

Chou WK, Dick S, Wararchuk WW, Tanner ME. Identification and characterization of NeuB3 from *Campylobacter jejuni* as a pseudaminic acid synthase. J Biol Chem 2005;280:35922–35928. [PubMed: 16120604]

Colegio OR, Griffin IV TJ, Grindley NDF, Galan JE. In vitro transposition system for efficient generation of random mutants of *Campylobacter jejuni.* J Bacteriol 2001;183:2384–2388. [PubMed: 11244083]

Doig P, Kinsella N, Guerry P, Trust TJ. Characterization of a posttranslational modification of *Campylobacter* flagellin: identification of a sero-specific moiety. Mol Microbiol 1995;19:379–387. [PubMed: 8825782]

Dorrell N, Mangan JA, Laing KG, Hinds J, Linton D, Al-Ghusein H, Barrell BG, Parkhill J, Stoker NG, Karylshev AV, Butcher PD, Wren BW. Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. Genome Res 2001;11:1706–1715. [PubMed: 11591647]

Elvers KT, Park SF. Quorum sensing in *Campylobacter jejuni*: detection of a *luxS* encoded signaling molecule. Microbiol 2002;148:1475–1481.

Fouts DE, Mongodin EF, Mandrell RE, Miller WG, Rasko DA, Ravel J, Brinkac LM, DeBoy RT, Parker CT, Dougherty SC, Dodson RJ, Durkin AS, Madupu R, Sullivan SA, Shetty JU, Ayodeji MA, Shvartsbeyn A, Schatz MC, Badger JH, Fraser CM, Nelson KE. Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. PLOS Biology 2005;3:e15. [PubMed: 15660156]

Frick IM, Morgelin M, Bjorck L. Virulent aggregates of *Streptococcus pyogenes* are generated by homophilic protein-protein interactions. Mol Microbiol 2000;37:1232–1247. [PubMed: 10972839]

Galdiero F, Romanao-Carratelli C, Nuzzo I, Bentivoglio C, Galdiero M. Phagocytosis of bacterial aggregates by granulocytes. Eur J Epidemiol 1988;4:456–460. [PubMed: 3203726]

Golden NJ, Acheson DWK. Identification of motility and autoagglutination *Campylobacter jejuni* mutants by random transposon mutagenesis. Infect Immun 2002;70:1761–1771. [PubMed: 11895937]

Goon S, Kelly JF, Logan SM, Ewing CP, Guerry P. Pseudaminic acid, the major modification on *Campylobacter* flagellin, is synthesized via the Cj1293 gene. Mol Microbiol 2003;50:659–671. [PubMed: 14617187]

Guerry P, Alm RA, Power ME, Logan SM, Trust TJ. Role of two flagellin genes in *Campylobacter* motility. J Bacteriol 1991;173:4757–4764. [PubMed: 1856171]

Guerry P, Doig P, Alm RA, Burr DH, Kinsella N, Trust TJ. Identification and characterization of genes required for post-translational modification of *Campylobacter coli* VC167 flagellin. Mol Microbiol 1996;19:369–378. [PubMed: 8825781]

Guerry P, Ewing CP, Hickey TE, Prendergast MM, Moran AP. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. Infect Immun 2000;68:6656–6662. [PubMed: 11083778]

Guerry P, Szymanski CM, Burr DH, Prendergast M, Ewing CP, Hickey TE, Pattarini D, Moran AP. Phase variation of *Campylobacter jejuni* 81–176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. Infect Immun 2001;70:787–793. [PubMed: 11796612]

Guerry, P., Yao, R., Alm, R. A., Burr, D. H., and Trust, T. J. (1994) Systems of experimental genetics for *Campylobacter* sp. *In* Methods in Enzymol.235: Bacterial pathogenesis, part A, Identification and regulation of virulence factors. Academic Press, San Diego, California.

Hickey TE, Majam G, Guerry P. Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytolethal distending toxin. Infect Immun 2005;73:5194–5197. [PubMed: 16041038]

Hildebrand M, Waggoner LE, Liu H, Sudek S, Allen S, Anderson C, Sherman DH, Haygood H. An unusual modular polyketide synthase gene from the uncultivated bacterial symbiont of themarine Bryozoan *Bugula neritina.* Chem Biol 2004;11:1543–1552. [PubMed: 15556005]

Jeon B, Itoh K, Misawa N, Ryu S. Effects of quorum sensing on *flaA* transcription and autoagglutination in *Campylobacter jejuni.* Microbiol Immunol 2003;47:833–839. [PubMed: 14638994]

Karlyshev AV, Linton D, Gregson NA, Wren BW. A novel paralogous gene family involved in phase variable flagella-mediated motility in *Campylobacter jejuni.* Microbiol 2002;148:473–480.

Karlyshev AV, Wren BW. Development and application of an insertional system for gene delivery and expression in *Campylobacter jejuni.* Appl Environ Microbiol 2005;71:4004–4013. [PubMed: 16000815]

Kirn TJ, Lafferty MJ, Sandoe CMP, Taylor RK. Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae.* Mol Microbiol 2000;35:896–910. [PubMed: 10692166]

Kirn TJ, Taylor RK. TcpF is a soluble colonization factor and protective antigen secreted by El Tor and Classical O1 and O139 *Vibrio cholerae* serogroups. Infect Immun 2005;73:4461–4470. [PubMed: 16040956]

Knutton S, Shaw RK, Anantha RP, Donnenberg MS, Zorgani AA. The type IV bundle-forming pilus of enteropathogenic *Escherichia coli* undergoes dramatic alterations in structure associated with bacterial adherence, aggregation and dispersal. Mol Microbiol 1999;31:499–509. [PubMed: 10027967]

Labigne-Roussel A, Harel J, Tompkins L. Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni.* J Bacteriol 1987;169:5320–5323. [PubMed: 2822671]

Larsen JC, Szymanski C, Guerry P. *N*-linked protein glycosylation is required for full competence in *Campylobacter jejuni* 81–176. J Bacteriol 2004;186:6508–6514. [PubMed: 15375132]

Lior H, Woodward DL, Edgar JA, Larouche LJ, Gill P. Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. J Clin Microbiol 1982;15:761–768. [PubMed: 7096555]

Linton D, Karlyshev AV, Hitchen PG, Morris HR, Dell A, Gregson NA, Wren BW. Multiple *N*-acetyl neuraminic acid synthetase (*neuB*) genes in *Campylobacter jejuni*: identification and characterization of the gene involved in sialylation of lipooligosaccharide. Mol Microbiol 2000;35:1120–1134. [PubMed: 10712693]

Logan SM, Kelly JF, Thibault P, Ewing CP, Guerry P. Structural heterogeneity of carbohydrate modifications affects serospecificity of campylobacter flagellins. Mol Microbiol 2002;46:587–597. [PubMed: 12406231]

Misawa N, Blaser MJ. Detection and characterization of autoagglutination activity by *Campylobacter jejuni.* Infect Immun 2000;68:6168–6175. [PubMed: 11035721]

Nataro JP, Kaper JB, Robins-Browne R, Prado V, Vial P, Levine MM. Patterns of adherence of diarrheagenic *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect Immun 1987;60:2297–2304. [PubMed: 1350273]

Oelschlaeger TA, Guerry P, Kopecko DJ. Novel microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii.* Proc Natl Acad Sci USA 1993;90:6884–6888. [PubMed: 8341714]

Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, Jagels K, Karlyshev AV, Moule S, Pallen MJ, Penn CW, Quail MA, Rajandream MA, Rutherford KM, van Vliet AH, Whitehead S, Barrell BG. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature 2000;403:665–8. [PubMed: 10688204]

Pearson BM, Pin C, Wright J, I'Anson K, Humphrey T, Wells JM. Comparative genome analysis of *Campylobacter jejuni* using whole genome microarrays. FEBS Lett 2004;554:224–230. [PubMed: 14596944]

Penner JL, Hennessy JN. Passive hemaglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. J Clin Microbiol 1980;12:732–737. [PubMed: 6796598]

Power M, Guerry P, McCubbin WD, Kay CM, Trust TJ. Structural and antigenic characteristics of *Campylobacter coli* FlaA flagellin. J Bacteriol 1994;176:3303–3313. [PubMed: 7515043]

Roggenkamp A, Neuberger HR, Flugel A, Schmoll T, Heesemann J. Substitution of two histidine residues in YadA protein of *Yersinia enterocolitica* abrogates collagen binding, cell adherence and mouse virulence. Mol Microbiol 1995;16:1207–1219. [PubMed: 8577254]

Schembri MA, Christiansen G, Klemm P. FimH-mediated autoaggregation of *Escherichia coli.* Mol Microbiol 2001;41:1419–1430. [PubMed: 11580845]

Schirm M, Schoenhofen I, Logan SM, Waldron K, Thibault P. Identification of unusual bacterial glycosylation by tandem mass spectrometry analysis of intact proteins. Anal Chem 2005;77:7774–7782. [PubMed: 16316188]

Schmidt MA, Riley L, Benz I. Sweet new world: glycoproteins in bacterial pathogens. Trends Microbiol 2003;45:267–561.

Schoenhofen IC, McNally DJ, Vinogradov E, Whitfield D, Young M, Dick S, Wararchuk WW, Brisson JR, Logan SM. Functional characterization of dehydratase/aminotransferase pairs from *Helicobacter* and *Campylobacter*: enzymes distinguishing the pseudaminic acid and bacillosamine biosynthetic pathways. J Biol Chem 2006;281:723–732. [PubMed: 16286454]

Sherlock O, Vejborg RM, Klemm P. The TibA adhesin/invasin from enterotoxigenic *Escherichia coli* is self-recognizing and induces bacterial aggregation and biofilm formation. Infect Immun 2005;73:1954–1963. [PubMed: 15784535]

Skurnik M, Bolin I, Heikkinen H, Piha S, Wolf-Watz H. Virulence plasmid-associated autoaglutination in *Yersinia* spp. J Bacteriol 1984;158:1031–1036.

Soo EC, Aubry AJ, Logan SM, Guerry P, Kelly JF, Thibault P. The selective detection and identificationof sugar-nucleotides by CE-ESMS and its application to bacterial metabolomics. Anal Chem 2004;76:619–626. [PubMed: 14750855]

Szymanski CM, Wren BW. Protein glycosylation in bacterial mucosal pathogens. Nat Rev Microbiol 2005;3:225–237. [PubMed: 15738950]

Thibault P, Logan SM, Kelly JF, Brisson JR, Ewing CP, Trust TJ, Guerry P. Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. J Biol Chem 2001;276:34862–34870. [PubMed: 11461915]

Vann WF, Silver RP, Abeijon C, Chang K, Aaronson W, Sutton A, Finn CW, Lindner W, Kotsatos M. Purification, properties, and genetic location of *Escherichia coli* cytidine 5'-monophosphate N-acetylneuraminic synthetase. J Biol Chem 1987;262:17556–17562. [PubMed: 2826425]

Vendeville A, Winzer K, Heurlier K, Tang CM, Hardie KR. Making "sense" of metabolism: autoinducer-2, LuxS and pathogenic bacteria. Nat Rev Microbiol 2005;3:383–396. [PubMed: 15864263]

Wong KH, Skelton SK, Patton CM, Feeley JC, Morris G. Typing of heat-stable and heat-labile antigens of *Campylobacter jejuni* and *Campylobacter coli* by coagglutination. J Clin Microbiol 1985;21:702–707. [PubMed: 3998098]

Yao R, Alm RA, Trust TJ, Guerry P. Construction of new *Campylobacter* cloning vectors and a new mutational *cat* cassette. Gene 1993;130:127–130. [PubMed: 8344519]

Yao R, Burr DH, Doig P, Trust TJ, Niu H, Guerry P. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni* defective in invasion of eukaryotic cells: the role of flagella in invasion. Mol Microbiol 1994;14:883–893. [PubMed: 7715450]

Yao R, Guerry P. Molecular cloning and site-specific mutagenesis of a gene involved in arylsulfatase production in *Campylobacter jejuni.* J Bacteriol 1996;178:3335–3338. [PubMed: 8655516]

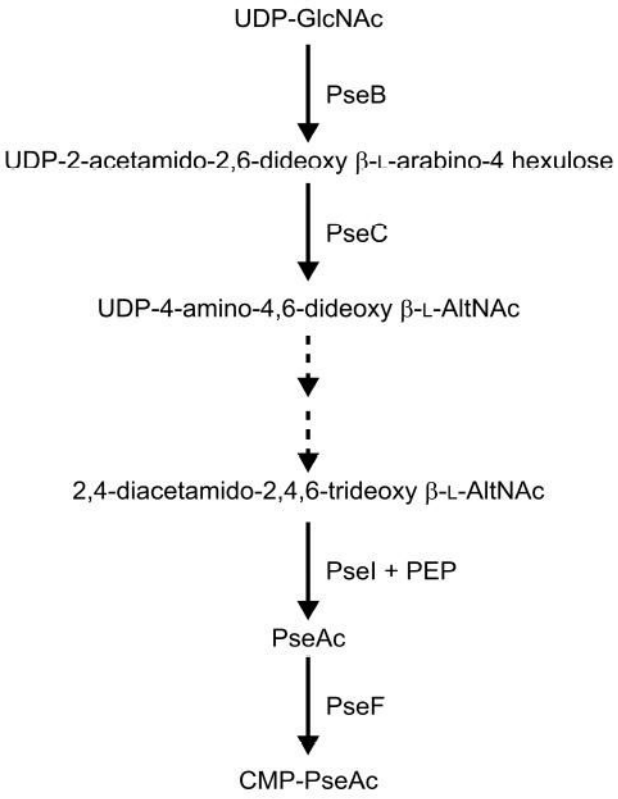

Fig. 1. Pathway of pseudaminic acid synthesis in *C. jejuni* from UDP-GlcNAc

The enzymatic activity of the products of PseB (Cj1293) and PseC (Cj1294) have been described in Schoenhofen *et al*. 2005 and that of PseI (Cj1317c) by Chou *et al.*, (2005). Broken arrows indicate biosynthetic steps for which the respective enzymatic activities have still to be assigned to a campylobacter gene. The presumed activity of PseF is based on homology to the corresponding enzyme in the sialic acid biosynthetic pathway, NeuA (Vann *et al.*, 1987).

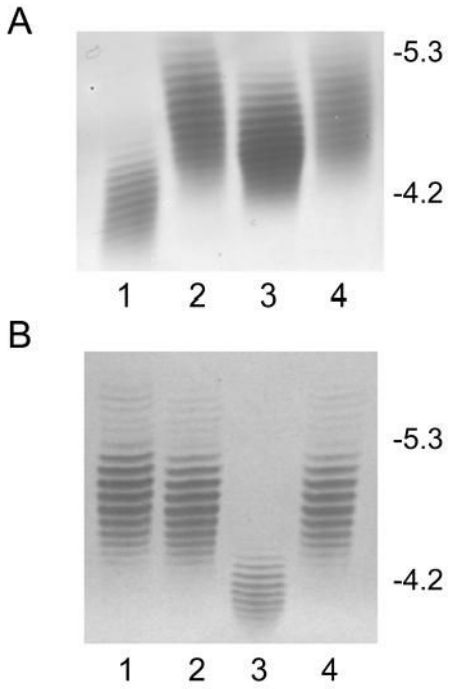

Fig. 2. Isoelectric focusing pattern of flagellins

Purified flagellins from 81–176 and various mutants were separated on an ampholyte mixture of pH 4–6 and stained with Gel Code Blue. A. Characterization of flagellin from the *pseD* mutant. Lane 1, 81–176 *pseA::cat* (Thibault *et al.*, 2001); lane 2, wildtype 81–176; lane 3, 81–176 *pseD::cat*; lane 4, 81–176 *pseD::cat* (pRY107/28+*pseD*). B. Complementation of *pseA* by insertion into *atsA*. Lane 1, 81–176 *atsA::aphA3*; lane 2, wildtype 81–176; lane 3, 81–176 *pseA::cat*; lane 4, 81–176 *pseA::cat*, *atsA::pseA+aphA3*. The position of IEF markers is indicated on the right.

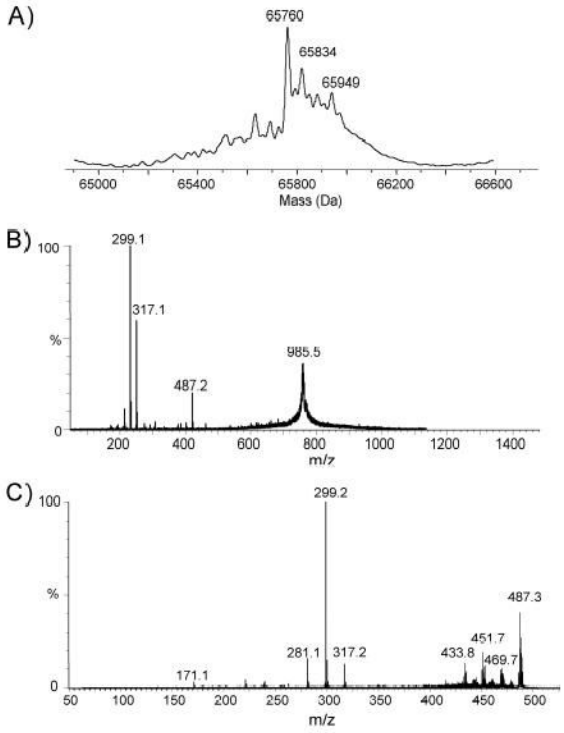

Fig. 3. Structural analysis of flagellin from a mutant in Cj1333

A. Molecular mass determination of intact flagellin from the mutant in *pseD*. B. Tandem mass spectrometry analysis of the multiply charged ion at m/z 985 from flagellin from the *pseD* mutant. C. Second generation product ion spectra of oxonium ion m/z 487 obtained from in source dissociation of multiply charged flagellin ion. Conditions: In-source fragmentation was obtained by increasing the RF Lens 1 from 50 to 125 V to produce oxonium fragment ions (m/z 487) from the native flagellin in the orifice/skimmer region. Argon was used as target gas at collision energy of 30 V.

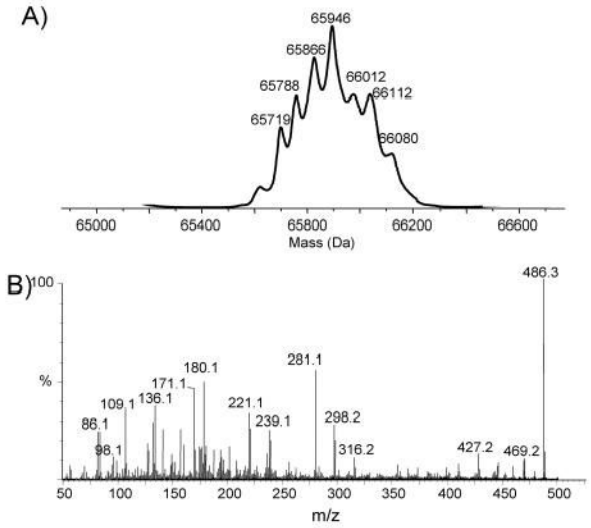

Fig. 4. Structural analysis of flagellin from 81–176 *pseD* (pRY107/*pseD*)

A. Molecular mass determination of intact flagellin from the complemented strain.

B. Second generation product ion spectra of oxonium ion m/z 486 obtained from in source dissociation of a multiply charged flagellin ion. The presence of m/z 316 product ion confirms the restoration of both groups on this flagellin (see Schirm *et al*., 2005). Conditions used for in source fragmentation are as described in figure 3.

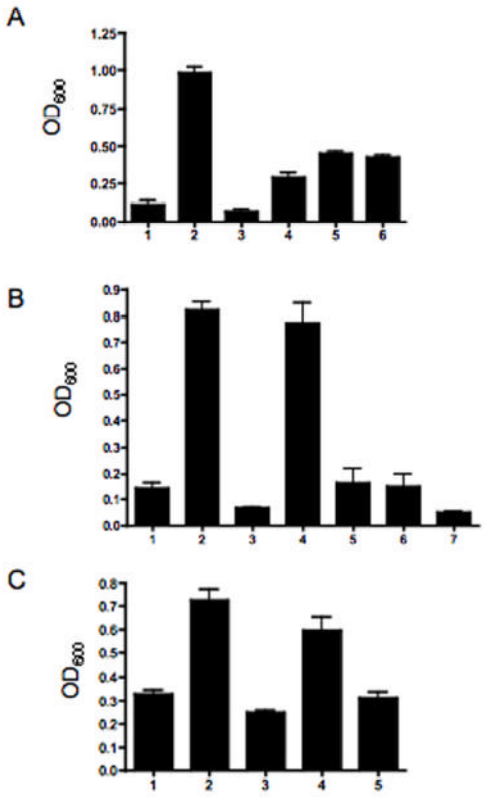

Fig. 5. Autoagglutination of *Campylobacters*

Suspensions of bacteria were set to an OD_{600} of 1.0 and incubated without shaking. After 18 h incubation at room temperature, the OD_{600} of the top 1 ml of the tube determined. Mutants used in this study are listed in Table 2. A. Comparison of wildtype 81–176 with isogenic mutants. Column 1, wild-type 81–176; column 2, 81–176 *flaA::aph3*; column 3, 81–176, *pflA::aph3*; column 4, 81–176 *kpsM::aph3*; column 5, 81–176 *neuC1::cat*; column 6, 81–176 *luxS::cat*. B. Flagellin glycosylation mutants of *C. jejuni* 81–176. Column 1, wild-type 81–176; column 2, 81–176 *pseD::cat*; column 3, 81–176 *pseD::cat* (pRY107/*pseD*); column 4, 81–176 *pseA::cat*; column 5, 81–176 *pseA::cat*, *astA::pseA*, *aph3*; column 6, 81–176 *astA::aph3*; column 7, 81–176 CjB1301::*cat*. C. Flagellin glycosylation mutants of *C. coli* VC167 T2. Column 1, wild-type VC167 T2; column 2, VC167 T2 *ptmD::aph3*; column 3, VC167 T1 *ptmD::aph3* (pRY111/*ptmD*); column 4, VC167 T2 *pseB::cat*; column 5, VC167 T2 *pseB::cat* (pRY107/*pseB*). The graphs show the mean and standard deviations of 2–8 independent determinations.

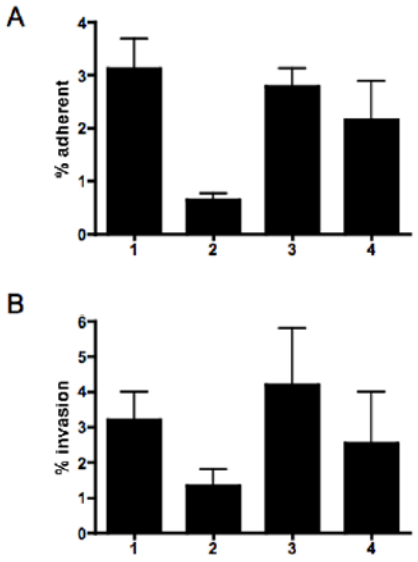

Fig. 6. The effect of loss of PseAm on adherence and invasion of INT407 cells

A. Adherence. B. Invasion. Column 1, wild-type 81–176; column 2, 81–176 *pseA::cat*; column 3, 81–176 *pseA::cat*, *astA::pseA+aphA3*; column 4, 81–176 *atsA::aphA3*.

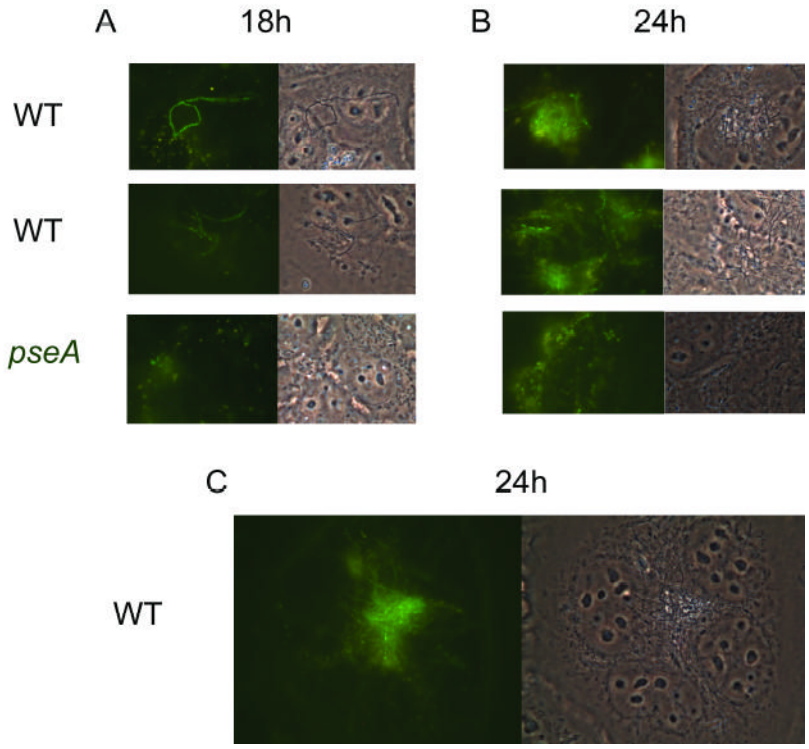

Fig. 7. Comparison of adherence patterns to INT407 cells of GFP tagged wildtype 81–176 with GFP tagged 1906 mutant

Panel A shows the adherence patterns of wildtype 81–176 and the *pseA* mutant following 18 h incubation. Panels B and C show adherence patterns following 24 h incubation. Fluorescence images are on the left and phase images are on the right. Final magnification of panels A and B, 1000X; final magnification of panel C, 1500X.

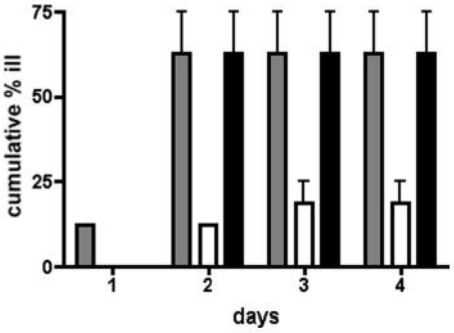

Fig. 8. Virulence in ferrets

Animals were fed *C. jejuni* at doses ranging from 3.8 x 10^{10}–4.5 x 10^{10} and monitored for diarrheal disease for 4 days. The data are presented as the cumulative % animals that developed diarrhea over time. Grey bars, 81–176; white bars, 81–176 *pseA::cat*; black bars, 81–176 *pseA::cat, astA::pseA + aph3*. The data represent the two separate experiments with 8 animals per group or a total number of 16 animals per strain.

Table 1

Genes in the flagellin glycosylation locus of *C. jejuni* 81–176.

NCTC homolog[1]	Gene/annotation[2]	Homology to 11168[3]	length bp	Position of insertion[4]	Phenotype of mutant	Reference
Cj1292	*dcd*; dCTP deaminase	98/99 (186)	561	N/A	ND	N/A
Cj1293	*pseB*	94/98 (334)	1005	395 NP	mot−	Goon *et al*., 2003
Cj1294	*pseC*	88/91 (376)	1131	654 NP	mot−	this work
Cj1295	amidopeptidase	95/97 (233)[5]	708	335 P	mot+; wt IEF	this work
Cj1300c	unknown	50/68 (295)	897	287 NP	mot+; wt IEF	this work
Cj1298	unknown	46/63 (234)	720	259 NP	mot+; wt IEF	this work
Cj1299	*accP*	72/85 (75)	228	ND	NA	NA
None (CJB1301)	NA	none	1359	807 NP/663 P	mot+; wt IEF	this work
Cj1302	unknown	95/95 (520)	1563	122 P	mot+; wt IEF	this work
Cj1303	*fabH2*	99/99 (353)	1062	302 NP	mot+; wt IEF	this work
Cj1304	*accP*	95/97 (73)	221	81 NP	mot+; wt IEF	this work
Cj1305c	unknown	81/86 (405)	1215	271 NP	mot+; wt IEF	this work
Cj1311	*pseF*	97/98 (232)	699	304 P	mot−	this work
Cj1312	*pseG*	92/92 (198)	597	304 P	mot−	this work
Cj1313	*pseH*	88/95 (127)	384	246 P	mot−	this work
Cj1314c	probable cyclase	93/93 (248)	747	134 NP	mot+; wt IEF	Thibault *et al*., 2001
Cj1315c	amidotransferase	98/99 (201)	606	205 NP	mot+; wt IEF	Thibault *et al*., 2001
Cj1316c	*pseA*	99/99 (378)	1137	NA	mot+; PseAm−	Thibault *et al*., 2001
Cj1317	*pseI*	92/93 (343)	1032	NA	mot−	Thibault *et al*., 2001
Cj1334	unknown *(maf3)*	71/84 (544)	1638	817 P	mot+; wt IEF	this work
Cj1333	*pseD*	64/79 (653)	1965	1407 NP	mot+/PseAm−; 486−; 487+	this work
Cj1337	*pseE*	61/76 (653)	1899	998 NP	mot−	this work
Cj1338c	*flaB*	75/81 (576)	1731	NA	N/A	this work
Cj1339c	*flaA*	75/81 (576)	1731	NA	N/A	NA
Cj1341c	unknown *(maf6)*	58/72 (605)	1827	1121 NP	mot+; wt IEF	this work
Cj1342c	unknown *(maf7)*	71/80 (404)	1251	1062 NP	mot+; wt IEF	this work

[1] The NCTC 11168 that showed the highest homology to 81–176 is listed; as stated in the text, some genes in 81–176 are in a different order than those in NCTC 11168;

[2] Annotations are the original annotations from Parkhill *et al.* (2000), Karlyshev *et al*., (2002) or from this work based on loss of PseAm or lack of motility suggesting a role in PseAc synthesis or attachment; annotations in parentheses indicate motility associated genes in NCTC 11168 but not 81–176;

[3] homology to NCTC11168 was done by blastp analysis at campyDB (http://campy.bham.ac.uk/) and is given at the % identity/% similarity (number of amino acids);

[4] position of the transposon insertion was determined by sequence analysis in *E. coli* and is given from the ATG start in bp;

[5] The 81–176 allele of Cj1295 was truncated due to a possible slip strand mismatch at a homopolymeric tract of As; if the slip strand was corrected, the predicted protein showed 94% identity/96% similarity to the NCTC 11168 gene over 418 amino acids.

Table 2

Other mutants used in this study.

Parent Strain	Genotype	Phenotype	Reference
C. jejuni 81–176			
	pseA::cat	lacks PseAm on flagellin	Thibault et al. (2001)
	pseA::cat, astA::pseA, aph3	*cis* complement of *pseA::cat*; AstA-	This study
	astA::aph3	AstA-	This study
	pseD::cat	lacks PseAm + 486 Da group on flagellin	This study
	pseD::cat (pRY107/*pseD*)	*in trans* complement of *pseD::cat*	This study
	flaA:: aph3	non-motile; non-flagellated	Yao et al. (1994)
	pflA::aph3	paralyzed flagella; non-motile	Yao et al. (1994)
	kpsM::aph3	non-encapsulated	Bacon et al. (2001)
	neuC::cat	lacks sialic acid on LOS	Guerry et al. (2001)
	luxS::cat	quorum sensing mutant	This study
C. coli VC167 T2			
	ptmD::aph3	lacks PseAm on flagellin	Logan et al. (2002)
	ptmD::aph3 (pRY111/*ptmD*)	*in trans* complement of *ptmD::aph3*	Logan et al. (2002)
	pseB::cat	lacks PseAc on flagellin	Goon et al. (2003)
	pseB::cat (pRY107/*pseB*)	*in trans* complement of *pseB::cat*	Goon et al. (2003)