L-fucose influences chemotaxis and biofilm formation in *Campylobacter jejuni*

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Key words: L-fucose, biofilms, chemotaxis, *Campylobacter jejuni*
Summary

*Campylobacter jejuni* and *Campylobacter coli* are zoonotic pathogens once considered
asaccharolytic, but are now known to encode pathways for glucose and fucose
uptake/metabolism. For *C. jejuni*, strains with the *fuc* locus possess a competitive advantage in
animal colonization models. We demonstrate that this locus is present in >50% of genome-
sequenced strains and is prevalent in livestock-associated isolates of both species. To better
understand how these campylobacters sense nutrient availability, we examined biofilm formation
and chemotaxis with fucose. *C. jejuni* NCTC11168 forms less biofilms in the presence of fucose,
although its fucose permease mutant (*fucP*) shows no change. In a newly developed chemotaxis
assay, both wild-type and the *fucP* mutant are chemotactic towards fucose. *C. jejuni* 81-176
naturally lacks the *fuc* locus and is unable to swim towards fucose. Transfer of the NCTC11168
locus into 81-176 activated fucose uptake and chemotaxis. Fucose chemotaxis also correlated
with possession of the pathway for *C. jejuni* RM1221 (fuc+) and 81116 (fuc-). Systematic
mutation of the NCTC11168 locus revealed that Cj0485 is necessary for fucose metabolism and
chemotaxis. This study suggests that components for fucose chemotaxis are encoded within the
*fuc* locus, but downstream signals, only in fuc+ strains, are involved in coordinating fucose
availability with biofilm development.
Introduction

*Campylobacter* infections, caused primarily by *C. jejuni* or *C. coli*, are among the leading causes of bacterial foodborne diarrheal disease (Allos, 2001; Silva *et al.*, 2011) and are associated with the development of Guillian-Barré syndrome and its variants (Taboada *et al.*, 2007; Keithlin *et al.*, 2014). *C. jejuni* and *C. coli* were previously considered to be asaccharolytic and rely on other carbon and nitrogen sources, such as amino acids and intermediates of the citric acid cycle for growth (Velayudhan and Kelly, 2002; Stahl *et al.*, 2012; Szymanski and Gaynor, 2012; Hofreuter, 2014). Recently, this dogma was challenged when *C. jejuni* NCTC11168 was reported to possess a *fuc* locus (*cj0480c-cj0490*) which encodes Cj0486, an L-fucose permease responsible for fucose transport across the inner membrane (FucP), and Cj0480c, the fucose operon repressor (FucR) along with other annotated, but uncharacterized genes required for L-fucose metabolism described in Table 1 (Muraoka and Zhang, 2011; Stahl *et al.*, 2011). Furthermore, it was recently shown that certain *C. coli* isolates possess the ability to transport and metabolize glucose (Vorwerk *et al.*, 2015).

Fucose is found in human and chicken mucin (Macfarlane *et al.*, 2005; Stahl *et al.*, 2011), on epithelial cell surfaces (Becker and Lowe, 2003; Pickard *et al.*, 2014; Wacklin *et al.*, 2014) and in our diet (Chaturvedi *et al.*, 2001; Chow and Lee, 2008; Zivkovic and Barile, 2011). *C. jejuni* binds to fucosylated structures (Ruiz-Palacios *et al.*, 2003; Day *et al.*, 2009) and this binding is inhibited by fucose-containing structures, such as fucosylated human milk oligosaccharides (Cervantes *et al.*, 1996; Ruiz-Palacios *et al.*, 2003; Newburg *et al.*, 2005; Weichert *et al.*, 2013). Furthermore, the presence of L-fucose offers an advantage to *C. jejuni* NCTC11168 by
enhancing its growth in laboratory media (Muraoka and Zhang, 2011; Stahl et al., 2011) and providing the wild-type with a competitive advantage in the piglet model of human disease over a \textit{fucP} mutant (Stahl et al., 2011). The wild-type also outcompetes the \textit{fucP} mutant at low infection doses in chickens fed with a fucose rich diet (Muraoka and Zhang, 2011). These findings suggest an important role for fucose binding, uptake and metabolism in \textit{Campylobacter} host colonization and pathogenesis.

Chemotaxis also plays an important role in the pathogenicity of \textit{C. jejuni}. Mutants in the chemotaxis signal transduction pathway (\textit{che}) are less virulent and exhibit reduced colonization in chicken and mouse colonization models (Hendrixson and DiRita, 2004; Chang and Miller, 2006), in addition to showing attenuation in the ferret disease model (Yao et al., 1997). Interestingly, L-fucose is the only carbohydrate chemoattractant for \textit{C. jejuni} (Hugdahl et al., 1988) and chemotaxis mutants, such as \textit{cheA} (Reuter and van Vliet, 2013), do not swim towards this sugar.

Biofilm formation is also associated with persistence and infection in several bacteria such as \textit{Pseudomonas aeruginosa}, \textit{Streptococcus pneumonia} and enteropathogenic \textit{Escherichia coli} (Costerton et al., 1999; Hall-Stoodley et al., 2004; Aparna and Yadav, 2008; Hall-Stoodley and Stoodley, 2009). \textit{C. jejuni} forms biofilms on epithelial cells (Haddock et al., 2010) and on abiotic surfaces (Trachoo et al., 2002; Kalmokoff et al., 2006; Sanders et al., 2008; Gunther and Chen, 2009; Nguyen et al., 2010; Moe et al., 2010; Maal-Bared et al., 2012) and is affected by multiple environmental factors. For example, \textit{C. jejuni} biofilm growth is reduced in the presence of NaCl and sucrose, and this is attributed to the osmotic stress induced by these compounds (Reeser et al., 2007). In addition, nutrient rich media, such as Brucella and Bolton broth, inhibit biofilms (Reeser et al., 2007) while the presence of organic materials and biofouling can increase biofilm
formation (Brown et al., 2014). Temperature and oxygen tension also alter biofilm densities (Reeser et al., 2007; Reuter et al., 2010). *C. jejuni* mutants with defects in biofilm formation show reduced chicken colonization and, adhesion and invasion of epithelial cells, as well as reduced intracellular survival (Svensson et al., 2009; Theoret et al., 2011; Theoret et al., 2012; Rahman et al., 2014).

In this study, we investigated the influence of the *fuc* locus on biofilm development and chemotaxis by *C. jejuni* NCTC11168. Biofilm formation by the wild-type is reduced in the presence of L-fucose, but remains unaltered in a *fucP* mutant. Transfer of the *fuc* locus genes (*cj0481-cj0490*) from NCTC11168 into the *fuc* locus deficient strain, 81-176, allowed the recombinant strain to actively transport L-fucose and enhanced its growth in the presence of this carbohydrate. Interestingly, we found that both *C. jejuni* NCTC11168 wild-type and the *fucP* mutant are chemotactic towards L-fucose and the transfer of the locus into 81-176 also triggered a positive chemotactic response towards this carbon source. The observed correlation between possession of the *fuc* locus and chemotaxis towards this carbon source allowed us to identify a product of the pathway, Cj0485 that links fucose metabolism and chemotaxis.

**Results**

**Distribution of the *fuc* locus in *C. jejuni* and *C. coli* genomes**

In our previous study, we reported that the *fuc* locus is present in *C. jejuni* strains NCTC11168, RM1221, CF93-6, 84–25, *C. jejuni* subsp. *doylei* 269.97, and *C. coli* RM2228, but absent in *C. jejuni* strains 81–176, CG8486, HB93-13, 260.94, and 81116 (Stahl et al., 2011). We have determined the prevalence of the *fuc* locus (*cj0480c-cj0490*) in 4,232 *C. jejuni* and *C. coli*
genome sequences, which were phylogenetically clustered using feature frequency profiling (van Vliet and Kusters, 2015). The fuc locus was present in 2,431 out of 3,746 C. jejuni genomes (64.9%) and 354 out of 486 C. coli genomes (72.8%) (Fig. 1, Table S1). The distribution of the fuc locus was associated with specific MLST-clonal complexes, such as ST-21, ST-48, ST-206, ST-354 and ST-257 in C. jejuni, while MLST-clonal complexes such as ST-45, ST-283, ST-42, ST-353 and ST-464 are mostly lacking the fuc locus (Fig. 1). In C. coli, the fuc locus was primarily found in the MLST-clonal complex ST-828, but not in the riparian isolates (Sheppard et al., 2013). Livestock-associated (agricultural) lineages are shown in red whereas water and wildlife-associated (environmental) lineages are shown in blue in Fig. 1 (Stabler et al., 2013). Interestingly, there appears to be a trend toward agriculture isolates possessing the pathway and environmental isolates lacking this pathway (Fig. 1), although the data set is currently skewed toward agriculture isolates linked with human infections.

**L-fucose modulates biofilm formation in C. jejuni NCTC11168**

We investigated biofilm formation in the presence of 25 mM L-fucose in static glass tube cultures. We found that addition of L-fucose to the MH culture medium caused an approximately 2-fold reduction in the amount of biofilm formed by wild-type C. jejuni NCTC11168 as determined by the absorbance at 570 nm via the crystal violet staining method (Fig. 2). The reduction was significantly different in a paired student’s t-test (p-value <0.05) (Fig. 2). Previously, a mutation in the fucose permease, fucP in this strain has been shown to inactivate L-fucose uptake from the extracellular environment (Muraoka and Zhang, 2011; Stahl et al., 2011). We found that biofilm formation by the fucP mutant in the presence of fucose was similar when compared to unsupplemented medium. To show that the observed biofilm change was specific to L-fucose, we assayed biofilm formation in the presence of 25 mM D-galactose. Biofilm
formation by the wild-type and \textit{fucP} mutant in media supplemented with D-galactose was not significantly different when compared to the amount of biofilm formed in unsupplemented media (Fig. 2). Our results also demonstrate that the amount of biofilm formation in unsupplemented media by wild-type \textit{C. jejuni} and the \textit{fucP} mutant are similar (Fig. 2). The fucose repressor mutant, \textit{fucR} exhibited a phenotype similar to wild-type and showed reduction in biofilm formation in medium supplemented with 25 mM L-fucose (Fig. S1).

We further investigated the effects of L-fucose on the appearance and architecture of biofilms by scanning electron microscopy (SEM). We analysed both \textit{C. jejuni} NCTC11168 wild-type and \textit{fucP} mutant biofilms that had formed on glass slides in MH or MH with 25 mM L-fucose. Wild-type \textit{C. jejuni} formed thick biofilms in the absence of 25 mM L-fucose (Fig. 3). These biofilms had a very dense mass of cells comparable to previous observations of \textit{C. jejuni} biofilms (Joshua \textit{et al.}, 2006; Brown \textit{et al.}, 2014) (Fig. 3). Interestingly, biofilm formation was severely reduced when cells were grown in the presence of 25 mM L-fucose with only a few \textit{C. jejuni} detectable on the glass slide (Fig. 3). In contrast, biofilm formation by the \textit{fucP} mutant appeared similar in density and architecture in the presence or absence of fucose. Biofilms formed by the \textit{fucP} mutant were also comparable to wild-type biofilms grown in the absence of L-fucose (Fig. 3). No biofilms were detected in the negative control samples. Our SEM analysis was consistent with the results obtained from the crystal violet staining assay.

\textbf{Transfer of the \textit{fuc} locus from \textit{C. jejuni} NCTC11168 into \textit{C. jejuni} 81-176}

\textit{C. jejuni} 81-176 naturally lacks the \textit{fuc} locus (Table S1) (Stahl \textit{et al.}, 2011). A plasmid containing \textit{cj0481} to \textit{cj0490} was constructed and transferred into 81-176 resulting in 81-176\textit{Ωfuc}. To test the functionality of the \textit{fuc} locus on the introduced plasmid, the uptake rates of $^{3}$H-L-fucose by 81-176\textit{Ωfuc} were determined in cells grown in the presence or the absence of...
fucose and compared to wild-type 81-176 and NCTC11168 that were grown under similar conditions. Wild-type NCTC11168 showed basal $^3$H-L-fucose uptake rates (3.3 pmol $^3$H-L-fucose/min/10$^9$ cfu) when grown in MH alone (Fig. 4A). $[^3]$H-L-fucose uptake rates significantly increased (around 4-fold to 12.0 pmol $^3$H-L-fucose/min/10$^9$ cfu) when wild-type cells were grown in the presence of fucose demonstrating the induction of the system in the presence of its substrate (Fig. 4A).

In contrast, no $^3$H-L-fucose transport was observed in 81-176 in the presence of fucose in the growth medium consistent with the absence of the fucose locus in this strain. (Fig.4A). Interestingly, 81-176Ωfuc exhibited significantly higher uptake rates of $^3$H-L-fucose independent of the presence or absence of fucose in the growth medium prior to the addition of $^3$H-L-fucose (34.8 pmol 3H-L-fucose/min 10$^9$ cfu and 36.5 pmol 3H-L-fucose/min/10$^9$ cfu, respectively), indicating that the pathway is constitutively expressed in this strain (Fig. 4A). Analysis of growth rates further demonstrated that C. jejuni 81-176 wild-type is unable to utilize L-fucose for enhanced growth. Growth curves and final OD$_{600}$ were similar in the presence or absence of this carbon source (Fig. 4B). In contrast, the C. jejuni 81-176Ωfuc strain grown in the presence of L-fucose showed significantly enhanced growth when compared to growth in MEM alone (Fig. 4B) (p-value <0.05). Similarly, wild-type C. jejuni NCTC11168 showed significant enhanced growth in MEM+L-fucose compared to MEM alone (Fig. 4B).

We also examined biofilm formation by C. jejuni 81-176 and 81-176Ωfuc and observed that biofilm formation in both strains was unaffected by supplementation with L-fucose. D-galactose was included as another carbohydrate control and did not affect biofilm formation in these strains (Supplementary Fig. 1).
Quantitative reverse transcriptase PCR (RT-PCR) was used to compare transcript levels of \(fucP\) in \(C. jejuni\) NCTC11168 and 81-176\(\Omega fuc\). FucP expression was significantly higher when \(C. jejuni\) NCTC11168 cells were grown in the presence of fucose compared to cells grown in unsupplemented MEM medium (Fig. 4C). Elevated \(fucP\) transcripts were detected at comparable levels when \(C. jejuni\) 81-176\(\Omega fuc\) was grown in MEM alone or in MEM+L-fucose (Fig. 4C). This indicates that the pathway is constitutively expressed in \(C. jejuni\) 81-176\(\Omega fuc\) and supports the observed increase and constitutive \(^3\)H-L-fucose uptake rates in this strain. We conclude that the \(fuc\) locus is fully functional in 81-176\(\Omega fuc\) and results in increased uptake of L-fucose and enhanced growth.

**Identification of fucose utilization genes that play important roles in enhanced growth of \(C. jejuni\) in L-fucose**

We also tested whether mutations in the \(C. jejuni\) NCTC11168 fucose utilization genes \(cj0484\), \(cj0485\) and \(cj0488\) affect growth in MEM alone or in MEM supplemented with L-fucose (Fig. 5), while other mutants in this locus have been previously analysed (Stahl et al., 2011). In agreement with previous observations, the wild-type strain showed approximately 2-fold enhanced growth in MEM supplemented with L-fucose (p-value <0.05) whereas the \(fucP\) mutant showed similar growth in MEM regardless of fucose addition (Fig. 5) (Muraoka and Zhang, 2011; Stahl et al., 2011). We found that the \(cj0485\) mutant did not show enhanced growth in the presence of L-fucose and the OD\(_{600}\) was similar when compared to unsupplemented medium. However, growth was enhanced in the presence of L-fucose after complementation of the \(cj0485\) mutation *in trans*. The \(cj0488\) mutant behaved similar to wild-type (p-value <0.05) (Fig. 5). The \(cj0484\) mutant also showed enhanced growth in the presence of fucose, however this increase was not significantly different compared to growth in MEM alone (Fig. 5). To eliminate the possibility of downstream
effects of the \textit{cj0485} mutation on \textit{cj0486} expression, we performed RT-PCR on \textit{fucP} in the \textit{cj0485} mutant and we were able to confirm expression of \textit{fucP} in this strain. 16s rRNA used as a control showed similar RNA levels in the samples (Fig. 5).

\textbf{The roles of the \textit{fuc} locus in chemotaxis towards L-fucose}

To examine \textit{C. jejuni} chemotaxis towards L-fucose, we initially used the PBS agar plate assay that is typically used for the analysis of chemotaxis in this species (Hugdahl \textit{et al.}, 1988; Khanna \textit{et al.}, 2006; Vegge \textit{et al.}, 2009; Baserisalehi and Bahador, 2011). However, this assay resulted in false positives when applied to our negative controls, chemotaxis and motility mutants, similar to that described in another study (Kanungpean \textit{et al.}, 2011).

To circumvent these problems, we established and confirmed the reproducibility of a novel assay with chemotaxis (\textit{cheY}) and flagellar mutants (\textit{flaA}). We further validated our assay using the previously described \textit{C. jejuni} NCTC11168 chemoattractants: L-serine, L-aspartate and L-fucose (Hugdahl \textit{et al.}, 1988; Vegge \textit{et al.}, 2009; Baserisalehi and Bahador, 2011) as well as PBS (solvent control) and L-histidine (a known non-attractant) as negative controls. The assays were set-up as described in Materials and Methods and in Fig. 6. No rings were observed in any tubes containing the \textit{flaA} and \textit{cheY} mutants (Fig.6 and Fig. S2). This indicated that our assay does not give false positives and had been established successfully. In addition, we only detected culturable \textit{C. jejuni} in the top layer of the chemotaxis tubes that showed positive results indicating that no passive diffusion of cells occurred and that the presence of cells is due to active migration through the agar as a chemotactic response towards the added substrate (data not shown).

We applied the assay to examine the chemotactic responses of the \textit{C. jejuni fucP} mutant. Red rings were observed around the positive control compound L-serine. Red rings were also
observed in the test tube containing L-fucose indicating that this strain is still chemotactic towards this compound (Fig. 6). Next we analysed the chemotactic responses of *C. jejuni* 81-176 and *C. jejuni* 81-176Ωfuc. Interestingly, 81-176Ωfuc was strongly chemotactic towards L-fucose as indicated by dark red rings around L-fucose in the tube whereas no red rings near L-fucose were observed for the parent 81-176 (Fig. 6). Consistent with published reports, 81-176 was chemotactic towards L-serine (Hugdahl et al., 1988; Baserisalehi and Bahador, 2011; Reuter and van Vliet, 2013) and the recombinant strain, 81-176Ωfuc, showed a similar chemotactic behaviour towards this amino acid. To further investigate the correlation between the presence of the *fuc* locus and fucose chemotaxis, we analysed the chemotaxis responses of the *fuc* locus deficient strain *C. jejuni* 81116 and the *fuc* locus positive strain *C. jejuni* RM1221. We could show that strain RM1221 was chemotactic towards L-fucose while strain 81116 was not (Fig. 6), indeed suggesting a correlation between the presence of the *fuc* locus and the ability to swim towards L-fucose. In addition red rings were observed around L-serine with RM1221 cells, but not with 81116 cells (Fig. 6). No red rings were observed with PBS for all the tested strains. The unusual observation that 81116 was naturally not chemotactic towards L-serine was further verified with the conventional chemotaxis plate assay (Fig. S3). Here no measurable chemotactic response towards L-serine could be observed for 81116 whereas *C. jejuni* NCTC11168 showed a strong chemotactic response towards this compound.

Next we investigated if the loss of specific *fuc* genes involved in L-fucose metabolism has an impact on the chemotaxis response towards L-fucose. Mutation in *cj0481*, *cj0483*, *cj0484*, *cj0487*, *cj0488* and *cj0490* did not affect the ability of *C. jejuni* to swim towards L-fucose or L-serine (Fig. S2). Interestingly, the *cj0485* mutant completely lost the ability to swim towards L-fucose (Fig. 6), but was still capable of swimming toward the L-serine positive control. This
indicates that the *cj0485* gene product is crucial for chemotaxis specifically towards L-fucose in *C. jejuni* NCTC11168. Upon complementation of the *cj0485* gene *in trans*, the strain regained the ability to swim towards L-fucose (Fig. 6). In order to determine if chemotaxis towards L-fucose is dependent on the metabolic breakdown product of Cj0485 or directly on the protein, we created the *cj0484/cj0486* double mutant that is deficient in both annotated L-fucose transporters thereby eliminating any possibility of L-fucose uptake and metabolism. We still observed chemotaxis in this strain (Fig. S4), suggesting that metabolic intermediates are not involved in chemotaxis.

We further examined whether a mutation in *cj0485* affected biofilm formation in response to L-fucose and observed that the *cj0485* mutant behaved similar to the *fucP* mutant in the test tube assay. We found that this mutant formed biofilms regardless of the presence of L-fucose (Fig. 2). As expected, biofilm formation by the *cj0485* mutant was also unaffected in the presence of D-galactose (Fig. 2). Complementation of the mutant *in trans* did not show any differences.

**Discussion**

The L-fucose uptake and utilization locus (*fuc, cj0480-cj0490*) in *C. jejuni* NCTC11168 provides the strain with a competitive advantage in avian and animal colonization models (Stahl et al., 2011; Muraoka and Zhang, 2011). From recent studies it is becoming apparent that *C. jejuni* and *C. coli* have lineage-specific distribution patterns of metabolic markers, such as the vitamin B5 biosynthesis cluster (Sheppard et al., 2013) and the *fuc* locus investigated in this study. The distribution of the *fuc* locus was previously suggested to be restricted to specific multilocus sequence types of *C. jejuni* and *C. coli* (de Haan et al., 2012), and we have confirmed and extended these observations here using a large collection of genome sequences from these
species. The *fuc* locus is nearly universally present in the clonal complexes ST-21, ST-48, ST-206, ST-257 and ST-354, which includes the reference isolates NCTC11168 and RM1221 used in this study, whereas the *fuc* locus is absent in other major lineages such as ST-42, ST-45 and ST-283, which includes other reference isolates such as 81116 and 81-176 used here (Fig. 1, Table S1). In *C. coli*, the majority of ST-828 isolates are positive for the *fuc* locus, while the riparian *C. coli* isolates lack the locus. It is not completely clear what causes the distribution pattern of the *fuc* locus, as many of the positive and negative livestock-associated isolates share the agricultural space and hence should have the opportunity for acquisition by horizontal gene transfer or natural transformation. Hence there may be other factors which govern the acquisition, functionality and maintenance of the *fuc* locus, rather than random exchange by natural transformation, such as the availability of fucose.

We found that the addition of L-fucose resulted in a 2-fold reduction in wild-type NCTC11168 biofilm formation in the standard crystal violet assay, whereas inactivation of the fucose permease, *fucP* abolished this phenotype. This indicates that active uptake of L-fucose is necessary to sense this carbon source. Examination of wild-type and *fucP* biofilms by SEM analysis showed that the biofilm architecture was similar to previously published reports (Joshua *et al.*, 2006; Kalmokoff *et al.*, 2006; Brown *et al.*, 2014), and showed a similar loss of biofilm formation with fucose, consistent with the crystal violet assay results. In many organisms, such as *Escherichia coli* and *Pseudomonas aeruginosa*, biofilm formation is tied to stress responses that can be induced by DNA damage, the presence of antibiotics at sub-inhibitory or high concentrations, and by extracellular metal ions (Landini, 2009). In *C. jejuni*, biofilm formation has been linked to extracellular stresses, such as oxidative and osmotic stress (Fields and Thompson, 2008; Svensson *et al.*, 2009). This study demonstrates that *C. jejuni* is also capable of
sensing nutrient availability and reacting by maintaining a larger proportion of cells in a
dispersed planktonic state. In C. jejuni isolates that are capable of fucose uptake and metabolism,
we propose that there exists an intracellular regulatory network that couples an external sensory
response with biofilm formation. It is likely that the inability to uptake/metabolize L-fucose by
the fucP mutant and starvation in the absence of L-fucose in the case of the wild-type in minimal
media may trigger a stress response that leads to higher levels of biofilm formation. This is
consistent with the findings that nutrient rich medium, such as Brucella and Bolton broth, inhibit
C. jejuni biofilm formation (Reeser et al., 2007). The observed phenomenon may maintain C.
jejuni in a planktonic lifestyle in the competitive environment of the intestinal tract, which may
consequently cause enhanced infections and efficient spread during diarrheal disease.

To further investigate the importance of the fuc pathway in C. jejuni, we transferred the fuc locus
cj0481-cj0490 from NCTC11168 into 81-176, a strain that is naturally fuc deficient. We found
that the fuc pathway is functional in the recombinant strain and results in active uptake of L-
fucose. Since the fuc locus was expressed on a plasmid, the copy number of the fuc genes in 81-
176 would be higher than in NCTC11168. In addition, the fuc locus repressor, fucR (cj0480)
(Stahl et al., 2011) was not included on the plasmid resulting in constitutive expression in 81-
176. This caused overall higher expression levels of the fuc genes in 81-176Ωfuc and
consequently ~3.5 fold higher uptake of L-fucose in the recombinant strain compared to
NCTC11168. However, similar to NCTC11168, L-fucose also enhanced the growth of the 81-
176Ωfuc strain indicating that the fuc locus encodes all the proteins that are required for uptake
and metabolism of L-fucose. Interestingly, our results also indicate that 81-176 lacks the
intracellular regulatory network to alter biofilm formation in response to L-fucose since this
compound had no influence on biofilm formation in wild-type 81-176 or the 81-176Ω*fuc recombinant strain (Fig. S1).

To confirm that the constitutive expression and higher copy numbers of the *fuc* locus from a plasmid in strain 81-176 are not influencing the biofilm phenotype, we constitutively expressed the *fuc* genes in NCTC11168 by creating a *fucR* regulatory mutant. The NCTC11168 *fucR* mutant showed a reduction in biofilm formation in the presence of fucose similar to the isogenic wild-type further supporting our proposal that isolates naturally expressing the fucose pathway also have coordinated sensing of this nutrient with biofilm formation.

During the analysis of the *fuc* mutants in the growth studies, we found that the *cj0485* mutant behaved like the *fucP* mutant and did not show enhanced growth in the presence of L-fucose indicating the product of this gene is involved in fucose metabolism. The complementation of the *cj0485* gene in this mutant restored the phenotype supporting our hypothesis. This mutant had also lost the ability to reduce biofilm formation in the presence of L-fucose, similar to a *fucP* mutant that is unable to uptake the substrate (Stahl *et al.*, 2011). However, this phenotype was not restored in complementation experiments possibly due to non-native levels of expression.

We also investigated the role of the *fuc* pathway in the chemotaxis response of *C. jejuni* NCTC11168. We established a new assay that eliminates false positive observations that have been reported in previous studies (Hugdahl *et al.*, 1988; Khanna *et al.*, 2006; Vegge *et al.*, 2009; Baserisalehi and Bahador, 2011). We confirmed that wild-type NCTC11168 is chemotactic towards L-fucose as described (Hugdahl *et al.*, 1988; Reuter and van Vliet, 2013), and show that the *fucP* mutant is chemotactic towards L-fucose. This suggests that fucose uptake is not required for chemotaxis. 81-176 encodes a functional chemotaxis pathway (Yao *et al.*, 1997) however it does not swim towards L-fucose. We found that the recombinant strain 81-176Ω*fuc*
displayed a strong chemotaxis response towards L-fucose. We also discovered that the *fuc* locus positive strain RM1221 was motile towards L-fucose, while the *fuc* locus deficient strain 81116 was not suggesting that components for fucose chemotaxis are encoded within the *fuc* locus. This led to the systematic analysis of the *fuc* mutants in the chemotaxis assay and the subsequent identification of *cj0485* as a link between fucose metabolism and chemotaxis. The *cj0485* mutant was unable to swim towards L-fucose, but complementing the gene *in trans* restored the phenotype. BlastP analysis of Cj0485 indicates that the protein is homologous to short chain dehydrogenase enzymes that are generally involved in metabolism of compounds such as carbohydrates, amino acids and lipids (Kavanagh *et al.*, 2008; Bijtenhoorn *et al.*, 2011). Recent findings have also implicated dehydrogenases in quorum sensing pathways in bacteria (Lord *et al.*, 2014). It is possible that *cj0485* encodes a protein that is involved in both fucose metabolism and sensing. The transducer-like proteins (Tlps), found in the cytoplasm and inner membrane of *C. jejuni*, are responsible for chemotaxis signaling (Zautner *et al.*, 2011). Type A Tlps transect the inner membrane to sense compounds in the periplasm whereas Types B and C receive cytoplasmic signals. Cj0485 does not have a predicted transmembrane domain and mutation of the fucose permeases does not result in a loss of chemotaxis, so we do not believe Cj0485 is a Tlp. However, the protein may be involved in coordinating the signal between Tlp-like proteins in the periplasm and the *che* pathway. Future research will focus on the characterization of this protein and its involvement in fucose chemotaxis.

Chemotaxis plays important roles in the pathogenicity of *C. jejuni*. Chemotaxis mutants are defective in chicken colonization and attenuated in the ferret diarrheal disease model (Yao *et al.*, 1997; Hendrixson and DiRita, 2004). *C. jejuni* exhibits chemotaxis towards many amino acids, salts of organic acids and purified mucin (Hugdahl *et al.*, 1988; Vegge *et al.*, 2009); however, L-
fucose is the only carbohydrate that serves as a chemoattractant for this pathogen. Since *C. jejuni* binds to fucosylated structures (Day *et al.*, 2009), the ability to sense fucose may be important for targeting sites for gut colonization which is inhibited by the addition of exogenous compounds such as fucosylated human milk oligosaccharides (Cervantes *et al.*, 1996; Ruiz-Palacios *et al.*, 2003; Newburg *et al.*, 2005; Weichert *et al.*, 2013). Furthermore the ability to metabolize fucose may be linked to virulence due to the competitive advantage observed for fucose-utilizing strains in a disease model (Stahl *et al.*, 2011) as well as the identification of *fucP* as a potential virulence factor (Javed *et al.*, 2010) and its overrepresentation in hyperinvasive strains (Fearnley *et al.*, 2008). These studies suggest that there may be a potential link between sensing and chemo-attraction towards L-fucose in *C. jejuni* thereby influencing pathogenicity.

Fucose is highly abundant in the intestine and plays an important role in the virulence of intestinal pathogens such as Enterohaemorrhagic *E. coli* (EHEC) and *Salmonella* Typhimium, (Robbe *et al.*, 2004; Pacheco *et al.*, 2012; Weichert *et al.*, 2013; Wang *et al.*, 2015). However, due to lack of secreted or surface exposed fucosidases, EHEC, *S. Typhimium*, as well as *C. jejuni*, are most likely unable to release fucose from commonly found oligosaccharides (Pacheco *et al.*, 2012). However, other members of the intestinal microbiota, such as *Bacteroides thetaiotaomicron*, possess multiple glycosidases and have been shown to provide free carbohydrates for EHEC, *S. Typhimium* and *Clostridium difficile* resulting in increased pathogenicity (Pacheco *et al.*, 2012; Ng *et al.*, 2013; Tailford *et al.*, 2015). Thus we predict that *C. jejuni* also scavenges fucose freed by microbiota-secreted fucosidases.

In this study, we report that the gene cluster for fucose uptake and metabolism is widespread among campylobacters. We demonstrate that this pathway is necessary for chemotaxis towards fucose and that fucose uptake influences biofilm formation. The new phenotypes described in
this study highlight the possible functions of the fuc locus in the persistence and severity of *C. jejuni* infections. Further investigations into fully characterizing the fuc locus may highlight potential targets for the treatment of *Campylobacter* infections, particularly if this pathway is associated with strains causing human infections.

**Experimental Procedures**

**Strains, plasmids and growth conditions**

*C. jejuni* strains were grown in MH broth (Difco™), MEM (Gibco) or on MH agar plates at 37°C under microaerobic conditions (85% N₂, 10% CO₂, 5% O₂). *E. coli* was grown on LB medium at 37°C under aerobic conditions. If required, antibiotics were added to a final concentration of 25 µg/mL for kanamycin and chloramphenicol, 100 µg/mL for trimethoprim and ampicillin, and 12.5 µg/mL for tetracycline. If not stated otherwise, L-fucose was added to a final concentration of 25 mM. Growth analysis of strains in the presence or absence of L-fucose was performed as described in (Stahl et al., 2011). Plasmids and oligonucleotides used in this study are listed in Table S2. Growth assays were performed as described earlier (Stahl et al., 2011).

**Identification of the fuc locus in *C. jejuni* and *C. coli* genome sequences**

A total of 3,746 *C. jejuni* and 486 *C. coli* genome sequences were obtained from public collections such as the *Campylobacter* pubMLST website (http://pubmlst.org/campylobacter/) (Jolley and Maiden, 2010) and Genbank (http://www.ncbi.nlm.nih.gov/genome/browse/), and are listed in Table S1 with accession numbers and assembly status. Genomes were searched using MIST (Kruczkiewicz et al., 2013) and the BLAST+ (v2.28) suite with each individual gene of the *C. jejuni* NCTC11168 fucose locus (cj0480c-cj0490). Genes were considered to be present if matching ≥ 90% with the query sequence. The MLST-clonal complex designation was
determined for all genomes using MIST, with the definition file provided by the *Campylobacter* pubMLST website. All genomes were provisionally annotated using Prokka (Seemann, 2014) and were also searched for the presence of the predicted proteins of the *fuc* locus using BLAST (Table S1). A phylogenetic tree of all genomes was constructed using Feature Frequency Profiling of whole genome sequences using a word length of 18 (van Vliet and Kusters, 2015), and the resulting tree was visualised using Figtree using the proportional setting for presentational purposes.

**Construction of the ΔcheY, ΔfucR, Δcj0484, Δcj0485, and Δcj0488 isogenic deletion mutants**

Construction of the isogenic deletion mutants was performed using the In-fusion Dry-down PCR cloning kit (Clontech). Briefly, the target gene plus flanking regions were amplified using Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) and the corresponding primers (Invitrogen) listed in Table S2. The In-fusion Dry-down cloning kit was used to directionally clone the amplified gene product into BamHI (Invitrogen) digested pUC19. Subsequently, inverse PCR was performed to amplify pUC19 plus the flanking end regions and part of the target gene. A chloramphenicol antibiotic resistance cassette was directionally cloned into the inverse PCR product, disrupting the target gene. The final construct was sequenced to confirm the absence of point mutations and then naturally transformed into *C. jejuni* NCTC11168. Clones were selected for on chloramphenicol supplemented MH agar plates and positive colonies were confirmed by PCR. Construction of an isogenic *C. jejuni* NCTC11168 *fucR* mutant was done as follows: a PCR reaction using chromosomal DNA of strain CjWM116a (*C. jejuni, fucR::cm*, Muraoka, 2011) was performed with oligonucleotides fucR-R and fucR-F. The obtained 1.4 kbp *fucR::cm* DNA fragment was purified and inserted into the chromosome of
C. jejuni NCTC11168 by natural transformation. Clones were selected and confirmed as described above.

**Complementation of the Δcj0485 mutant strain**

The C. jejuni NCTC11168 cj0485 gene was amplified using Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) and the corresponding primers (Invitrogen) listed in Table S2. The amplified cj0485 gene was directionally cloned into XbaI digested pRRK plasmid using the In-fusion Dry-down PCR cloning kit (Clontech). The pRRK+cj0485 construct was sequenced to confirm the absence of PCR-induced errors in the cj0485 gene. The final construct was naturally transformed into the C. jejuni NCTC11168 Δcj0485 mutant strain and successful transformants were selected for on MH plates containing chloramphenicol and kanamycin. Insertion of the cj0485 gene was confirmed by PCR.

**Biofilm assay**

Campylobacter cells were grown in 5 ml of MH broth with required antibiotics for 18 hrs at 37°C under microaerobic conditions (85% N₂, 10% CO₂, 5% O₂) with shaking. Cultures were adjusted to an OD₆₀₀ of 0.05 and supplemented with 25 mM L-fucose or 25 mM D-galactose in MH broth. One millilitre of culture was subsequently added to borosilicate test tubes (13 x100 mm, Fisher Scientific) and supplemented with either L-fucose, D-galactose or MH alone. Test tubes containing only MH broth or MH broth supplemented with either L-fucose or D-galactose were used as negative controls. The tubes were sealed and further incubated at 37°C under microaerobic conditions for five days without shaking. Cultures were removed and the tubes were stained by coating with 100 µl of 1% crystal violet in 95% ethanol for 20 minutes at room temperature. The crystal violet stain was rinsed off thoroughly with distilled water until the wash
was clear. Biofilms were dislodged by adding 500 µl of 2% SDS in water and vortexing until a homogenous solution was formed. One hundred microlitres of the solution was transferred into a 96-well plate and the absorbance at 570 nm was measured in a plate reader. The crystal violet absorbance of the negative control tubes was subtracted from the absorbance readings of the other samples. A student’s paired t-test analysis was performed using Excel software (Microsoft®) and a p<0.05 was considered statistically significant.

**Scanning electron microscope (SEM) of C. jejuni biofilms on glass**

Cells were grown as described above (biofilm assay). One millilitre of each culture with an OD$_{600}$ of 0.05 was transferred into 24 well plates containing 10x20 mm glass slides (Thomas Scientific). After 5 days of incubation without shaking, supernatants containing planktonic cells were removed. Biofilms formed on the glass slide were fixed with 2 mL of fixative reagent (2.5% glutaraldehyde, 2 % paraformaldehyde in 0.1 M phosphate buffer) at 4°C until the samples were processed. The slides were washed three times for 10 min with 0.1 M phosphate buffer (PBS, pH7.5). The biofilm samples were sequentially dehydrated for 10 min in 50% ethanol, 70% ethanol, 90% ethanol, 2 × 100% ethanol, 75:25 ethanol: hexamethyldisilazane (HMDS), 50:50 ethanol: HMDS, 25:75 ethanol: HMDS, 100% HMDS and dried overnight in the fume hood. The slides were mounted on an SEM stub for coating with gold using a Nanotech SEMPrep 2 DC sputter coater. The EOL 6301F field emission scanning electron microscope was used for the SEM with a liquid nitrogen cooled lithium drifted silicon energy dispersive x-ray (EDX) detector with a Norvar window manufactured by PGT.

**Transfer of the fuc locus from C. jejuni NCTC11168 into 81-176 and analysis of [3H]-L-fucose uptake**
The fuc locus spanning the genes cj0481 to cj0490 (but lacking, cj0480/fucR) was amplified from C. jejuni 11168 chromosomal DNA as follows: 3883 bp and 4964 bp fragments, both including a native EcoRI restriction site, were amplified from chromosomal DNA with primers CS618-CS619 and CS620-CS621 and Pfx polymerase (Invitrogen). Both fragments were digested with EcoRI and inserted into a three arm ligation reaction into plasmid pBluescriptKS+ linearized with EcoRV. Positive clones with insertion of the fuc operon in the orientation of the lacZ gene were confirmed by restriction analyses and named pBluescriptKS+ (fuc). Plasmid pBluescriptKS+ (fuc) was subsequently digested with EcoRV-Xhol and the 8633 bp DNA fragment (containing cj0481 to cj0490) was purified and inserted into the E. coli - Campylobacter shuttle vector pCE111-28 (Larsen et al., 2004) treated with the same enzymes. Formation of the correct ligation product was screened and confirmed by restriction analyses. Plasmid DNA from one positive clone was named pCE111-28 (fuc) and used to transform E. coli C600 (RK212.2). E. coli C600 (RK212.2) (fuc) cells were used to conjugate the pCE111-28 (fuc) plasmid into C. jejuni 81-176 wild-type cells as described (Yao et al., 1997). Chloramphenicol resistant colonies were selected on MH plates supplemented with chloramphenicol and the presence of the plasmid was confirmed after plasmid-DNA isolation from 81-176Ωfuc and restriction analyses. [3H]-L-fucose uptake was performed as described previously (Stahl et al., 2011) with strains C. jejuni NCTC11168, C. jejuni NCTC11168 fucP, C. jejuni 81-176 wt and C. jejuni 81-176Ωfuc.

Reverse transcriptase PCR (RT-PCR)

Analysis of fucP mRNA transcripts was performed as described by Muraoka and Zhang (2011) with primers Cj0486 RT For and Cj0486 RT Rev for amplification of cj0486 and 16s RT For and 16s RT Rev (Table S2) for amplification of 16s rRNA internal control.
Tube-based chemotaxis assay

Chemotaxis assays were performed as follows: 500 μL of cells (2.8 mL per gram of cell pellet) in 0.4% PBS-agar were transferred to the bottom of a 2 mL Eppendorf tube and allowed to solidify for 30 min at room temperature. Samples were overlaid first with 100 μL of PBS agar that was allowed to set for 30 min, followed by 900 μL of 0.4% PBS agar and allowed to solidify for an additional 30 min at room temperature. A sterile piece of Whatman paper, soaked with 50 μL of a 1 M solution of L- fucose, L-serine, or 1xPBS was placed on top and samples were incubated under microaerobic conditions for 72 hrs at 37°C. Active bacterial cells that migrated through the upper layer of PBS-agar towards the compound added to the Whatman paper were visualised by adding 500 μL of 0.01% 2,3,5 triphenyltetrazolium chloride (TTC) in PBS. The respiratory dye TTC detects redox activity from active bacterial cells and results in formation of red rings of bacterial cells that are visible after 3-4 hr incubation under microaerobic conditions (Brown et al., 2013; Reuter and van Vliet, 2013). In addition, plating of the accumulated bacteria from the top layer of the agar confirmed the presence/absence of viable cells that migrated from the bottom of the tube towards the substrate on the top.

Plate-based chemotaxis assay

C. jejuni strains 81116 and 11168 were cultured under microaerobic conditions at 37°C overnight in MH broth. Strains were centrifuged at 6000 rpm at 4°C for 10 min and washed with 1X PBS. The strains were then centrifuged, resuspended to an OD600 of 2 and mixed at a 1:1 ratio with 0.8% PBS agar (final concentration of OD600 of 1, 0.4% PBS agar). Fifteen millilitres of each strain was poured into Petri dishes and allowed to solidify. Six millimeter paper absorbancy disks soaked with 1 M L-serine or PBS (negative control) were placed on the surface of the agar.
The strains were incubated for 24 hours under microaerobic conditions at 37°C and the diameter of chemotaxis was measured (mm).

Acknowledgements

We would like to thank Arlene Oatway (Biological Sciences Microscopy Facility, University of Alberta), and Nathan J. Gerein and George D. Braybrook (Earth and Atmospheric Sciences Microscopy Facility, University of Alberta) for their assistance with scanning electron microscopy, Qijing Zhang (Iowa State University) for providing chromosomal DNA of strain CjWM116a, and Kofi Garbrah for assistance with the graphical abstract. RD holds a Queen Elizabeth II Graduate Scholarship, CMS is an AITF iCORE Strategic Chair in Bacterial Glycomics. JG received a NSERC Alexander Graham Bell Canada Graduate Scholarship and an Alberta Innovates Graduate Student Scholarship. AHMvV is supported by the Biotechnology and Biological Sciences Research Council (BBSRC) via the BBSRC Institute Strategic Programme (BB/J004529/1). This publication made use of the PubMLST website (http://pubmlst.org/) developed by Keith Jolley and cited at the University of Oxford. The development of that website was funded by the Wellcome Trust. We are also grateful to the contributors of genome sequences available from the Campylobacter pubMLST website. AS acknowledge funding from CIHR (MOP#84224).

References


Table 1: Putative functions and mutant phenotypes of proteins encoded by the fucose locus

<table>
<thead>
<tr>
<th>Protein Number</th>
<th>Putative Function</th>
<th>Mutant Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cj0480c</td>
<td>fucose operon repressor / transcriptional regulator</td>
<td>nd</td>
<td>This study</td>
</tr>
<tr>
<td>Cj0481</td>
<td>dihydrodipicolinate synthase</td>
<td>no</td>
<td>Stahl et al. 2011; this study</td>
</tr>
<tr>
<td>Cj0482</td>
<td>altronate hydrolase / dehydratase</td>
<td>mutant has not been constructed</td>
<td>N/A</td>
</tr>
<tr>
<td>Cj0483</td>
<td>altronate hydrolase / dehydratase</td>
<td>yes</td>
<td>Stahl et al. 2011; this study</td>
</tr>
<tr>
<td>Cj0484</td>
<td>MFS transporter</td>
<td>limited</td>
<td>This study</td>
</tr>
<tr>
<td>Cj0485</td>
<td>short chain dehydrogenase</td>
<td>no</td>
<td>This study</td>
</tr>
<tr>
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<td>MFS transporter</td>
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<td>Stahl et al. 2011; this study</td>
</tr>
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<td>no</td>
<td>Stahl et al. 2011; this study</td>
</tr>
<tr>
<td>Cj0488</td>
<td>epimerase</td>
<td>yes</td>
<td>This study</td>
</tr>
<tr>
<td>Cj0489</td>
<td>aldehyde dehydrogenase</td>
<td>yes</td>
<td>Stahl et al. 2011; this study</td>
</tr>
<tr>
<td>Cj0490</td>
<td>aldehyde dehydrogenase</td>
<td>mutant has not been constructed</td>
<td>N/A</td>
</tr>
</tbody>
</table>

nd: not determined; - no/low biofilm formation in the presence of fucose (similar to the wildtype); + normal biofilm formation in the presence of fucose.

*only tested as double-mutant with cj0486

Figure Legends

Fig. 1. Prevalence of the fucose operon among 3,746 C. jejuni and 486 C. coli genomes. Genomes were phylogenetically clustered using FFPr feature frequency profiling with L=18 (van Vliet & Kusters, 2015), and the resulting phylogenetic tree has been transformed using the "proportional" setting of Figtree for presentational purposes. The first row labeled 'fucose' indicated genomes possessing the fucose pathway, which are shown in red while those lacking the pathway are shown in black. The second bar shows the primary combinations of MLST clonal complexes for C. jejuni and C. coli, with red-labeled clonal complexes representing livestock-associated lineages, blue-labeled clonal complexes representing water/wildlife-
associated lineages as described by Stabler et al (2013), with the exception of ST-61 which has been described as livestock-associated in other studies (Kwan et al, 2008; Rotariu et al, 2009). The association of some clonal complexes such as ST-464 was not reported previously, and these are in black font. The lowercase letters indicate the approximate position of strains 81116 (a), 81-176 (b), RM1221 (c) and NCTC11168 (d).

Fig. 2. The effect of L-fucose on C. jejuni NCTC11168 biofilm formation A) Absorbencies of solubilized biofilm obtained from the crystal violet assay measured at 570 nm. P-value of <0.001 obtained in a paired student’s t-test is indicated by an asterisk (*). The bars represent an average of technical triplicates and the data is representative of at least 6 biological replicates. Error bars indicate standard error. B) Test tubes with crystal violet stained biofilms formed under the indicated conditions, (-) indicates growth in MH broth alone, (F) indicates growth in MH broth in the presence of 25 mM L-fucose, (G) indicates growth in MH broth in the presence of 25 mM D-galactose.

Fig. 3 Scanning electron microscopy of C. jejuni NCTC11168 biofilms on glass slides formed under the indicated conditions in MH broth at 1000x magnification. Negative control indicates MH broth with no bacterial cells. The images are representative of three independent experiments.

Fig. 4 Functional analysis of the C. jejuni NCTC11168 fuc locus (cj0481- cj0490) after transfer into C. jejuni 81-176. A) Uptake of [3H]- L-fucose in the indicated strains including C. jejuni 81-176 and C. jejuni 81-176 (fuc). B) Growth of indicated strains in minimal essential medium in
the presence (black bars) or absence of L-fucose (white bars). C) Reverse transcriptase PCR analysis of \textit{fucP} in the indicated strains grown in minimal essential medium with (black bars) or without L-fucose (white bars). P-value of <0.05 in a student’s paired t-test is indicated by an asterisk. Bars indicate average value obtained from at least three independent experiments and error bars indicate standard error.

Fig. 5 Growth analysis of indicated \textit{C. jejuni} NCTC11168 wild-type and specific \textit{fuc} mutant strains in minimal essential medium supplemented with (black bars) or without L-fucose (white bars). The bars represent values obtained from at least two independent experiments and error bars indicate standard error. A student’s paired t-test was performed and an asterisk represents a p-value < 0.05. The inset shows reverse transcriptase PCR (RT-PCR) of 16s rRNA and \textit{cj0486} mRNA in the wild-type \textit{C. jejuni} NCTC11168 strain and the indicated mutants. Negative control lane contains no mRNA.

Fig. 6 Analysis of the chemotaxis response of the indicated \textit{C. jejuni} strains. A) A schematic of the chemotaxis assay. B) Images of the chemotaxis assay tubes of the indicated strains after development with 0.01% TTC (2,3,5 triphenyltetrazolium chloride). The + and – signs indicate the presence or absence of chemotaxis towards the compounds. The images are representative of at least three independent experiments.
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Supplementary data for:

L-fucose influences chemotaxis and biofilm formation in *Campylobacter jejuni*

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This file includes:

**Figure S1** – Effect of L-fucose on biofilm formation by *C. jejuni* NCTC 11168 fucose repressor mutant (*fucR*) and 81-176.

**Figure S2** – Chemotaxis results of *C. jejuni* NCTC 11168 fucose locus mutants that are not chemotactic towards fucose.

**Figure S3** – *C. jejuni* 81116 is not chemotactic towards serine.

**Figure S4** – Chemotaxis assay of the *C. jejuni* NCTC 11168 Δcj0484/Δcj0486 fucose permease double mutant.
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A) Absorbances of solubilized biofilm obtained from the crystal violet assay measured at 570 nm. The bars represent an average of technical triplicates and error bars indicate standard error.

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Fig. S2. Chemotaxis results of *C. jejuni* NCTC 11168 fucose locus mutants that are not chemotactic towards fucose. The assay was performed as described in Figure 6. The + and – signs indicate the presence or absence of chemotaxis towards the compounds and images are representative of at least three independent experiments.
Fig. S3. *C. jejuni* 81116 is not chemotactic towards serine. Plate-based (A) and tube-based (B) assays were performed using *C. jejuni* NCTC 11168 as a positive control as described in Materials and Methods.
Fig. S4. Chemotaxis assay of the *C. jejuni* NCTC 11168 Δcj0484/Δcj0486 fucose permease double mutant. Chemotaxis to L-fucose was examined in this background to eliminate the possibility that fucose could enter through the alternate annotated permease.