Evaluating the Role of an Outer Membrane Lipoprotein Cj1279c in the Pathogenesis of *Campylobacter jejuni*

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ABSTRACT

Campylobacter jejuni is considered to be the most common bacterial cause of human gastroenteritis worldwide, and is often acquired from contaminated poultry products. However, fundamental aspects of its pathogenesis remain poorly understood. Proteomic technology and bioinformatic analysis have identified a number of membrane-associated proteins including lipoproteins in C. jejuni. Among those proteins is (designated Cj1279c), which seems to be involved in the interaction of C. jejuni with the host cell surface and contribute to pathogenesis. Cj1279c contains at least three fibronectin type III domains, which often associated with cell surface binding. Importantly, it has been found to be required for chicken colonization in vivo and efficient binding to LMH chicken cells in vitro. The Cj1279c gene is highly conserved in eight C. jejuni genome sequences. The aim of this study was to explore its contribution to Campylobacter adhesion to human cells by using Cj1279c mutants in an existing cell-association assay (Caco-2 cells). Cj1279c mutants were also examined in association and ELISA assays. The Cj1279c mutants did not show a significant reduction in adherence to the human enteric-like cell line Caco-2 as well as to fibronectin. This finding may eliminate the involvement of this lipoprotein in the pathogenesis of C. jejuni and suggest a different role for Cj1279c in chicken and human hosts. Further work is required to examine the binding of Cj1279c to other potential ligands by performing adhesion and invasion assays.

Key words: *Campylobacter jejuni*, Pathogenesis, Cj1279c, Lipoprotein, Fibronectin, FlpA

INTRODUCTION

Campylobacter jejuni are gram negative, micro-aerophilic, spiral or curved-shaped flagellated bacteria [1]. According to the world health organisation (WHO), "Campylobacter is 1 of 4 key global causes of diarrhoeal diseases. It is considered to be the most common bacterial cause of human gastroenteritis in the world". Infection with *C. jejuni* within a period of 1-10 days (usually 3-6 days) results in a wide range of diseases including diarrhoea, abdominal cramps, headache, fever, myalgia and malaise, which can be accompanied by the presence of blood in the stools [2]. Furthermore, *C. jejuni* infection has been associated with serious clinical complications such as Guillain-Barré Syndrome (GBS), pancreatitis, meningitis and urinary tract infections [3,4].

C. jejuni colonizes the gut of many animals particularly chickens, dogs and rodents in an asymptomatic manner [5,6]. The most common routes of transmission to humans are via raw meat (chicken), milk and contaminated water [2]. It is reported that the highest rate of transmission in the UK and the US occurs in the summer due to barbequed

chicken and other environmental sources [5,7]. *C. jejuni* strains have several mechanisms and virulence factors allowing them to effectively colonize and harm their potential hosts. Firstly, they are attracted to appropriate mucosal layers by chemotaxis and cell motility [8]. The absence of these elements decreases *C. jejuni* colonization of the mucosal layers of the gastro intestinal tract [9,10]. Typically, to colonize, invade epithelial cells and avoid excretion by peristalsis, a pathogenic microorganism like *C. jejuni* requires adherence factors [11]. There are well-characterised adhesins including outer membrane proteins, such as CadF, JlpA and Peb1 [12]. CadF is an outer membrane protein, which binds to fibronectin, which is a component of the intestinal cell membrane [11,13]. This fibronectin binding activity maps to amino acids 134-137 of CadF [11]. An additional adhesin is a lipoprotein, JlpA, which is exposed in the cell surface and is required for efficient Hep-2 and epithelial cells binding [12,14]. Binding to another putative adhesin, Peb1 (also called CBF1) is important for adherence and mutants without it colonized intestinal cells of an animal model poorly. CapA is an autotransporter adhesin and a study done on CapA-deficient mutants revealed decreased colonization and persistence in chickens as well as reduced adherence to human Caco-2 cells in vitro [12].

Investigations using proteomic technology have identified a number of novel membrane-associated proteins (including lipoproteins) in *C. jejuni* NCTC 11168 [15]. Prior analysis of such membrane associated lipoproteins using bioinformatics, has identified (designated Cj1279c), whose molecular features suggest it may be involved in the interaction of *C. jejuni* with host cells and hence contribute to pathogenesis [16]. Cj1279c contains at least three fibronectin type III domains, which have often been associated with cell surface binding and a signal sequence (N-terminal) with a prokaryotic membrane lipoprotein attachment site [17]. A researcher group has named Cj1279c Fibronectin-like protein A (FlpA) and confirmed that it acts as an adhesin to chicken epithelial cells, and is required for efficient chicken colonization in vivo [18]. Therefore, *C. jejuni* mutants lacking this gene should be tested in an existing cell-association assay using Caco-2 cells to confirm whether Cj1279c is a novel adhesin to human cells. The aim of this study was to evaluate the potential contribution of Cj1279c to *Campylobacter* adhesion to human Caco-2 cells.

METHODS

Bacterial strains

To propagate mutagenic constructs, *Escherichia coli* JM109 strain was used as a host. This strain was grown in Luria-Bertani agar/broth (LB) at 37°C. Ampicillin (100 µg/ml) and kanamycin (50 µg/ml) were added if required. *Campylobacter jejuni* NCTC 11168 wild type and mutant derivatives were cultured on Mueller-Hinton agar/broth or CCDA (charcoal-cefoperazone-deoxycholate agar) at 42°C under microaerophilic conditions (3% H_2, 6% O_2, 5% CO_2, 86%), with or without kanamycin (50 µg/ml) according to requirements.

Generation of *C. jejuni* null mutants

C. jejuni NCTC 11168 was electrotransformed according to the method of Wassenar's et al (1993) [19]. Briefly, in order to obtain electrocompetent *C. jejuni* cells, the overnight growth from one agar plate was harvested into 10 ml MHB and centrifuged at 7000 g for 10 min. The cells were resuspended in 20 ml of ice-cold W&E buffer (15% glycerol, 272 mM sucrose). This washing was repeated three times, before the cells were finally resuspended in 1ml W&E buffer. To electroporate the competent *C.jejuni* cells, 5 µg of plasmid was added to an electroporation cuvette (0.2 cm inter-electrode-distance; Bio-Rad) containing 40 µl of the competent *C. jejuni* 11168 cells and placed on ice for 10 min. The cells were then subjected to electroporation in a Bio-Rad Gene Pulser at a voltage of 2.5 kV, a capacitance of 25 µFD and a resistance of 200 Ω. Following electroporation, the cells were resuspended in 0.1ml of SOC medium before being spread out onto antibiotic-free CCDA plates. An overnight incubation at 42°C allowed the recovery of the transformants, which were then harvested, resuspended in 0.1ml MHB and plated out onto MH agar containing kanamycin. After 2-3 days, well-separated colonies were examined by PCR to confirm the correct integration of the mutagenic constructs.

Growth and adhesion assays

Growth curve assays were performed on *C. jejuni* NCTC_11168 wild type and Cj1279c mutants. Both strains were grown overnight in 20 ml MHB. The next day, the bacteria were then diluted until OD600 became 0.2 and measured after 60 min. The OD reading was measured every 2 h for 7 h and then one final reading taken after 24 h. Colonic Caco-2 cells were grown on DMEM (Dulbecco's MOD Eagle Medium Gibco) containing 10% or 2% foetal calf/bovine serum (FCS/FBS) with or without 1% antibiotic-antimyocotic (penicillin and streptomycin) at 37°C.

Association assays were performed using Ashgar et al.'s 2004 method [20]. Caco-2 cells were grown to confluence in DMEM containing 10% foetal calf serum (FCS) and 1% antibiotic-antimycotic solution) in 24-well tissue culture plates (2 cm2, Costar®, Corning Inc.). The next day, the media (DMEM 10% FCS) on the Caco-2 cells was replaced with 1 ml DMEM containing 2% FCS without the antibiotic-antimycotic solution. While *C. jejuni* strains were grown overnight in 20 ml MHB. The following day, the bacteria were harvested by centrifugation and resuspended in DMEM + 2% FCS. Cultures were equilibrated and approximately 1 x 108 cfu of bacteria (subsequently confirmed by retrospective serial dilution and counting) were added to each well in a volume of 0.5 ml. Bacteria were incubated with the Caco-2 cells at 37°C for 2 h with 5% CO2. Following incubation, monolayers were washed three times in pre-warmed 1 x PBS. Monolayers were then disrupted by the addition of 1 ml 0.5% sodium deoxycholate (in PBS) Serial 10-fold dilutions were made in 1 x PBS and 10 µl aliquots were plated out onto charcoal plates and incubated for two days at 42°C.

ELISA

DIG-labelling *C. jejuni*

C. jejuni wild type strain and Cj1279c mutants were grown overnight on 10 ml MHB. They were centrifuged at 9000 g for 5 min before being resuspended in 10 ml PBS-T (Tween). This washing procedure was repeated twice followed by the addition of 2 µl digoxigenin (DIG) according to the OD600, readings of cultured cells. This was followed by 3 washes in PBS-T before resuspension in PBS-1% BSA (bovine serum albumin, Sigma) and diluting down to a theoretical OD of 0.04.

Coating the ELISA plate with fibronectin

Wells of a 96 well ELISA plate were coated with 100 µl of fibronectin (2 µg/ml) in filtered (0.2 µM) carbonate buffer (8 mM Na2CO3, 142 mM NaHCO3, pH 9.4). 100 µl PBS-1% BSA only was added into control wells before the plates being incubated with shaking for 3 h at room temperature. Next, the plate was washed three times

with PBS-T before being blocked with 100 ℤl of 1% BSA-PBS for 1 h. 100 ℤl of the DIG-labelled bacteria was added to the plate and incubated on a shaker at 4°C for 24 h. The following day, the plate was washed 5 times with PBS-T using an ELISA plate washer and tap dried. Then, 100 ℤl of anti-digoxigenin (HRP conjugate, Roche) in PBS-1% BSA (1 to 5000 diluted) was added followed by 1 h incubation at room temperature. It was then washed 5 times with PBS-T and tap dried. Finally, 100 ℤl of ABTS substrate (2,2'-AZINO-bis [3-ethylbenziazoline-6-Sulfonic acid]) (CHEICON INTERNATIONAL) was added into each well before the plate was read at 405 nm.

RESULTS

Growth curve assays of *C. jejuni* 11168 wild type and Cj1279c mutants

A series of bacterial growth assays was performed on *C. jejuni* 11168 wild type, *C. jejuni* Cj1279c null mutant. These assays confirmed that the deletion of Cj1279c has no impact on the in vitro growth mutants. Figure 1 shows that *C. jejuni* 11168 wild type and Cj1279c mutants grew at the same rate. All assays were repeated independently twice.
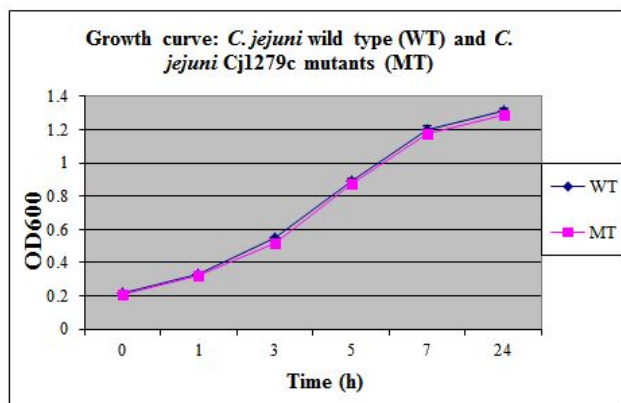

Figure 1: *C. jejuni* **11168 wild type (WT, blue line) and** *C. jejuni* **Cj1279c mutants (MT, pink line) showed no difference in growth under in vitro conditions.**

Association and ELISA assays

The Cj1279c mutant, FlaAB (*C. jejuni* 11168 mutant lacking flagellum and known to have a reduced ability to adhere and invade) and *C. jejuni* NCTC-11168 wild-type strains were tested for their ability to adhere to monolayers of Caco-2 cells. Colony counts for each strain confirmed that a similar inoculum of each strain was added to the adhesion assay (Figure 2). The mutant strain Cj1279c did not show any significant reduction in association to the human enterocyte-like cell line (P=0.25). In contrast, the control FlaAB mutant showed a significant reduction in association to Caco-2 cells compared to the wild type strain (P=0.01), (Figure 3). The experiment was repeated independently twice and a one-tailed t-test was used to analyse the means of the adherence levels of the wild type and the mutants.
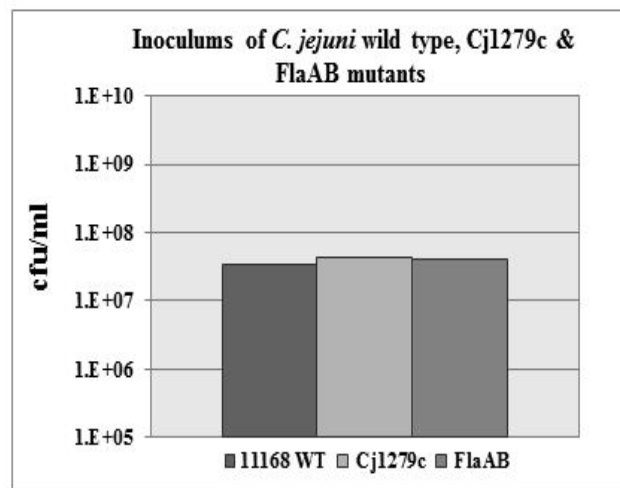

Figure 2: Comparison of inoculums used in the association assays. 11168 WT=*C. jejuni* **11168 wild type, Cj1279c=Cj1279c mutants, FlaAB=CjFlaAB mutants.**
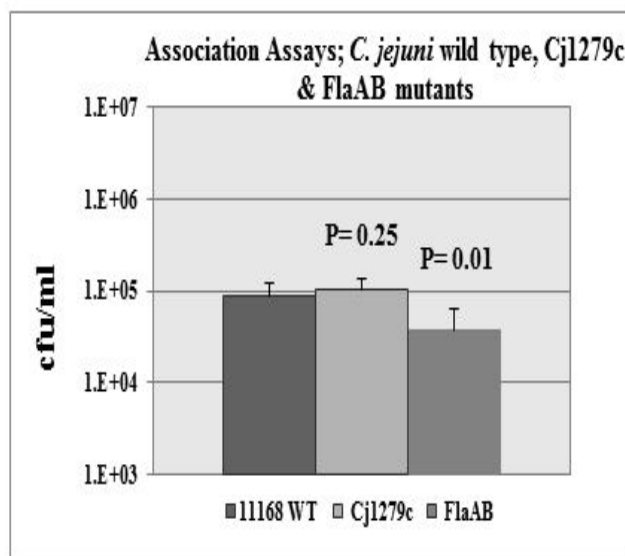

Figure 3: *In vitro***, adhesion tests using Caco-2 cells. Mutation of Cj1279c did not significantly affect association (P=0.25). In contrast, mutation of FlaAB (control) showed a significant reduction in association (P=0.01). 11168 WT=***C. jejuni* **11168 wild type, Cj1279c=Cj1279c mutants, FlaAB=CjFlaAB mutants.**

In addition to the adhesion assays, ELISA tests were used to examine the ability of wild-type cells and the Cj1279c mutant to bind to fibronectin.

Preliminary findings suggested that there was no significant difference between the wild type cells and the Cj1279c mutants in terms of binding to fibronectin (Figure 4).

The experiment was repeated independently three times and a one-tailed t-test was used to analyse the means of the adherence levels of the wild type and the mutants.
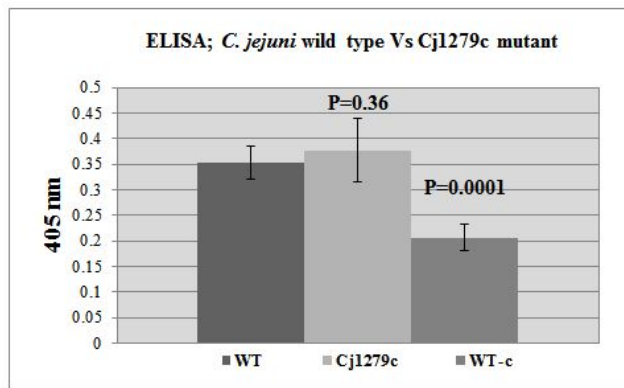
Figure 4: *In vitro* **ELISA assays using fibronectin. Mutation of Cj1279c did not significantly affect binding to fibronectin (P=0.36). Wild type cells added to fibronectin-free wells as a control showed a significant reduction (P=0.0001). WT=wild type, Cj1279c=Cj1279c mutants, WT-c=wild type control.**

DISCUSSION

In 2000, the genome sequence of *C. jejuni* NCTC-11168 wild type was completed [16]. This has enabled proteomic technology and bioinformatics analysis to identify a number of putative membrane-associated proteins, which may be involved in *Campylobacter* pathogenesis. Accordingly, one of the identified lipoproteins designated as Cj1279c is proposed to be involved in the interaction of *C.jejuni* with the host cell surface and hence in pathogenesis. Interestingly, Cj1279c has been termed fibronectin-like protein A (FlpA) after being found to be required for efficient cell adherence to chicken LMH cells and in vivo chicken colonisation [18]. Although the Cj1279*c* protein was found to be required for chicken colonization and efficient binding to LMH cells [18], mutants lacking this gene did not show a significant reduction in adherence to the human enteric-like cell line Caco-2. FlaAB mutants (negative controls known to have reduced ability to adhere and invade) were also examined in the same manner as Cj1279c and showed significantly reduced binding to Caco-2 cells compared with the wild type. This is surprising given the proposed role of Cj1279c (FlpA) in adhesion and colonisation in chickens, but if confirmed suggests that different adhesins may be involved in attachment to chicken and human cells. Similarly, CadF, CapA, JlpA and PEB1 mutants showed a significant reduction in in vitro binding to INT407, Caco-2, Hep-2 and HeLa human cells, respectively [14,19-22], only CadF and CapA had a similar impact on LMH chicken cells [18]. Moreover, only CadF and PEB1 were found to be essential for chicken colonization in vivo [18]. Based on these findings, it seems that some of the identified adhesins are present in their natural hosts in an asymptomatic manner whereas others are only required for pathogenesis, with the exception of CadF. Thus, for example, CadF binds to fibronectin [11,13] as does FlpA, yet the latter did not show any pathogenic characteristics in this study. Given the presence of the fibronectin-like domain in Cj1279c, an ELISA was designed to determine whether the Cj1279c mutant was deficient in binding to fibronectin in compared to the wild-type strain. The Cj1279c mutant was as efficient as wild-type strains in binding to fibronectin. In fact, the wild type and the Cj1279c mutant showed an almost identical level of binding to Caco-2 cells and to fibronectin. Although no difference was seen in association assays, this does not exclude a role of Cj1279c in invasion, which can be assessed using a gentamicin protection assay *in vitro*. Further work is required to validate these results and to examine the binding of Cj1279c to other potential ligands by repeating adhesion assays and performing invasion assays as well as more specific and sensitive tests like ELISA and far western blot. Similar lipoprotein, Cj0091 could go through the same process but using collagen protein instead of fibronectin in ELISA assays. If either mutant were found to be impaired in adherence, complementation would be crucial to prove that such influence was a direct result of the mutation and neither due to a polar effect nor a secondary mutation. In addition, two approaches could be used in order to investigate the surface localization of both lipoproteins on C. *jejuni*; flow cytometry and sub-cellular fractionation. Finally, since both genes are highly conserved in eight *Campylobacter* genome sequences, they may represent possible vaccine candidates for prevention of travel-associated diarrhoea caused by *C. jejuni*.

CONCLUSION

In conclusion, the Cj1279c mutant was examined in association and ELISA assays and was found to bind to Caco-2 cells in a similar manner to the wild-type strain. This suggests that the *Cj1279c* gene may not play a major role in binding to human epithelial cells despite being required for chicken colonization and efficient binding to LMH cells. As a result, further work is required to confirm or refute any role for this lipoprotein in adhesion to human cells. This study has excluded a potential virulence factor, however, further evaluation of this lipoprotein and similar molecules will contribute to a better understanding of the pathogenesis of *C. jejuni*.

REFERENCES

1. Hu L, Kopecko DJ. Interactions of *Campylobacter* with eukaryotic cells: Gut luminal colonization and mucosal invasion mechanisms. *Campylobacter*, 2nd Edn. ASM Press, Washington, DC. 2000; 191-215.

2. https://www.who.int/news-room/fact-sheets/detail/campylobacter

3. Skirrow MB. Clinical aspects of *Campylobacter* infection. *Campylobacter* 2000; 69-88.

4. Moore JE, Corcoran D, Dooley JS, et al. Campylobacter. Vet Res 2005; 36:351-82.

5. https://www.scirp.org/(S(i43dyn45teexjx455qlt3d2q))/reference/ReferencesPapers.aspx?ReferenceID=1418907

6. Skirrow MB. Epidemiology of Campylobacter enteritis. Int J Food Microbiol 1991; 12:9-16.

7. Bradbury J. *Campylobacter jejuni roots uncovered. Lancet Infect Dis 2005; 5:745.*

8. Hugdahl MB, Beery JT, Doyle M. Chemotactic behavior of Campylobacter jejuni. Infection Immunity 1988; 56:1560-6.

9. Konkel ME, Monteville MR, Rivera-Amill V, et al. The pathogenesis of *Campylobacter jejuni*-mediated enteritis. Curr Issues Intest Microbiol 2001; 2:55-71.

10. Guerry P. Campylobacter flagella: Not just for motility. Trends Microbiol 2007; 15:456-61.

11. Konkel ME, Christensen JE, Keech AM, et al. Identification of a fibronectin-binding domain within the Campylobacter jejuni CadF protein. Mol Microbiol 2005; 57:1022-35.

12. Young KT, Davis LM, DiRita VJ. Campylobacter jejuni: Molecular biology and pathogenesis. Nature Rev Microbiol 2007; 5:665-79.

13. Monteville MR, Konkel ME. Fibronectin-facilitated invasion of T84 eukaryotic cells by Campylobacter jejuni occurs preferentially at the basolateral cell surface. Infection Immunity 2002; 70:6665-71.

14. Jin S, Joe A, Lynett J, et al. JlpA, a novel surface-exposed lipoprotein specific to Campylobacter jejuni, mediates adherence to host epithelial cells. Mol Microbiol 2001; 39:1225-36.

15. Cordwell SJ, Len AC, Touma RG, et al. Identification of membrane-associated proteins from Campylobacter jejuni strains using complementary proteomics technologies. Proteomics 2008; 8:122-39.

16. Parkhill J, Wren BW, Mungall K, et al. The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. Nature 2000; 403:665-8.

17. Konkel ME, Larson CL, Flanagan RC. Campylobacter jejuni FlpA binds fibronectin and is required for maximal host cell adherence. J Bacteriol 2010; 192:68-76.

18. Flanagan RC, Neal-McKinney JM, Dhillon AS, et al. Examination of Campylobacter jejuni putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization. Infect Immunity 2009; 77:2399-407.

19. Wassenaar TM, Fry BN, van der Zeijst BA. Genetic manipulation of Campylobacter: Evaluation of natural transformation and electro-transformation. Gene 1993; 132:131-5.

20. Ashgar SS, Oldfield NJ, Wooldridge KG, et al. CapA, an autotransporter protein of Campylobacter jejuni, mediates association with human epithelial cells and colonization of the chicken gut. J Bacteriol 2007; 189:1856-65.

21. Monteville MR, Yoon JE, Konkel ME. Maximal adherence and invasion of INT 407 cells by Campylobacter jejuni requires the CadF outer-membrane protein and microfilament reorganization. Microbiol 2003; 149:153-65.

22. Kervella M, Pages JM, Pei Z, et al. Isolation and characterization of two Campylobacter glycine-extracted proteins that bind to HeLa cell membranes. Infect Immunity 1993; 61:3440-8.