Campylobacter jejuni PflB is required for motility and colonisation of the chicken gastrointestinal tract

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ABSTRACT

Campylobacter jejuni is the leading cause of foodborne bacterial gastroenteritis worldwide. Although the mechanisms by which *C. jejuni* causes disease are not completely understood, the presence of functional flagella appears to be required for colonisation of the gastrointestinal tract of humans and animals. Therefore much attention has been given to understanding the synthesis and role of flagella in *C. jejuni*. In this study we report insights into the function of PflB that is essential for *Campylobacter* motility. We have explored the function of this gene by constructing deletion mutants in *C. jejuni* strains NCTC11168 and M1, in the genes *cj0390* and *CJM1_0368*, respectively. The mutants were non-motile yet

assembled flagella that appeared structurally identical to the wild type. Furthermore the protein is required for *C. jejuni* colonisation of caeca in a two-week old chicken colonisation model.

Keywords: *Campylobacter jejuni*; flagella; motility

1. Introduction

Campylobacter spp. have emerged over the last three decades as significant clinical pathogens, responsible for a significant amount of bacterial gastroenteritis worldwide [1], where *Campylobacter jejuni* is thought to be responsible for 80-85% of all enteric *Campylobacter* infections [2]. Avian species are considered the main zoonotic reservoir for *C. jejuni* where the bacteria are considered to be a commensal. The most important route of human *Campylobacter* infection in industrialised nations is believed to be the consumption and handling of contaminated poultry [3]. Several post infection complications have been documented, where Guillain-Barré syndrome (GBS) is recognised as being one of the most serious, involving acute demyelination of the peripheral nervous system and ascending paralysis with potentially irreversible neurological damage [4].

C. jejuni possesses one or two polar flagella which enable motility, and also appear to be involved in cell adhesion, biofilm formation and secretion of proteins which mediate cell invasion [5-8]. The flagellar filament of some bacterial species bears strong antigenic properties and can be recognised by Toll-like receptor 5 (TLR5) [9]. *C. jejuni* flagellin possesses specific amino acid changes in the TLR5 recognition site that enables it to evade recognition [10]. *Campylobacter* flagellin is modified by *O*-linked glycosylation which may

47 influence the interaction of *C. jejuni* with host cells or play a role in immune evasion [11].
48 The major flagellin FlaA and its pseudaminic acid derivatives have been found to interact
49 with host Siglec-10, a glycan receptor, modulating the production of IL-10 *in vitro*. This may
50 aid bacterial colonisation *via* an anti-inflammatory strategy [12].

51 Flagella synthesis and function have been studied extensively in bacteria such as the
52 *Enterobacteriaceae* but it has become apparent that flagella systems in the
53 Epsilonproteobacteria, the class which includes *Campylobacter spp*., diverge from this
54 paradigm and possess novel components [13,14]. The *C. jejuni* flagella transcriptional
55 cascade involves the FlgS-FlgR two-component signal transduction system at the top of the
56 hierarchy which mediates expression of flagellar genes and ultimately flagella biosynthesis
57 [15-17]. Although much has come to light in terms of the structure and functions of the *C.*
58 *jejuni* flagellum, our knowledge is far from complete.

59 Previously, we generated *C. jejuni* signature tagged transposon mutants (STM) and
60 screened the mutants for defects in motility [18]. A transposon insertion within gene
61 NCTC11168 *cj0390* (*pflB*) was found to have a non-motile phenotype. In this study, we
62 report findings based on characterisation of NCTC11168 and M1 strains with a deletion in
63 this gene (*cj0390* and *CJM1_0368*, respectively).

64

65 2. Materials and methods

66 *2.1. Bacterial strains and growth conditions*

67 *C. jejuni* were cultured in Mueller-Hinton (MH) broth or on MH agar plates,
68 supplemented with 5% (v/v) defibrinated horse blood (Oxoid). Both plates and cultures
69 were incubated at 42˚C in microaerophilic conditions (5% v/v O_2, 5% v/v CO_2, 90% v/v N_2) in

70 a MACS-VA500 Variable Atmosphere Workstation (Don Whitley Scientific). *Escherichia coli*
71 DH5α were cultured in Luria Bertani (LB) media at 37˚C in liquid culture or on LB agar plates.
72 Media were supplemented with antibiotics where appropriate at final concentrations of:
73 trimethoprim, 5 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; chloramphenicol, 10 μg ml^{-1}; and
74 kanamycin, 35 μg ml^{-1}.
75

76 *2.2. Construction of directed gene deletion mutants*

77 PCRs were performed in 50μl volumes consisting of genomic DNA, (60-100ng) or
78 plasmid DNA (10-20ng), 0.5μM forward and reverse primers (Sigma), 200μM dNTPs, 2.5U
79 ProofStart DNA polymerase (Qiagen) and 1xProofStart reaction buffer. Reactions were
80 performed using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) as
81 follows: 4 mins at 94˚C, then 30 cycles of: 1 min at 94˚C, 1 min at 55˚C, 1 min at 72˚C
82 followed by a final extension of 7 mins at 72˚C. Standard protocols were used for molecular
83 cloning [19]. DNA was treated with restriction endonucleases, alkaline phosphatase and
84 ligated according to the manufacturer's instructions (Roche; New England Biolabs). Genomic
85 and plasmid DNA were prepared using the Genomic-tip Kit and QIAprep Spin Miniprep Kit,
86 respectively, according to the manufacturer's instructions (Qiagen). An inverse PCR strategy
87 was used to generate a defined NCTC11168 *cj0390* mutant by deleting 1520 bp of the gene
88 and by insertion of a chloramphenicol acetyl transferase (*cat*) cassette. *C. jejuni* NCTC11168
89 genomic DNA template and primers ak1 and ak2 (Table 1) were used to amplify *cj0390* with
90 the introduction of flanking *Kpn*I and *Bam*HI restriction sites. The PCR product was digested
91 using KpnI and BamHI and cloned into similarly digested pUC19 [20]; the resulting plasmid
92 was designated pAK3 (Table 2). Inverse PCR was performed using pAK3 as template and

primers ak9 and ak10 (Table 1) which introduced a unique *Xma*I site and a 1520 bp deletion of *cj0390*. *Xma*I-ended *cat* cassette, obtained from restriction digestion of plasmid pAV35 [21], was ligated to the inverse PCR product. The resulting plasmid was designated pAK4 (Table 2) and introduced into *C. jejuni* by natural transformation, generating NCTC11168 Δ*cj0390*a and M1 Δ*CJM1_0368*a. A second *C. jejuni* NCTC11168 Δ*cj0390* mutant, designated Δ*cj0390*b, was generated where *cj0390* was deleted in its entirety and replaced by a *cat* cassette. PCR was performed using primers ak47 and ak48 (Table 1) to amplify 922 bp of DNA directly upstream of NCTC11168 *cj0390.* This was cloned into the *Eco*RI-*Bam*HI restriction sites of pUC19 [20]; the resulting plasmid was designated pAK10 (Table 2). A *cat* cassette, PCR amplified from pRY107 [22] using primers ak51 and ak52, was cloned into the *Bam*HI-*Sal*I restriction sites of pAK10. This plasmid was designated pAK11. An 832 bp region of DNA, located immediately downstream of *cj0390*, was PCR amplified using primers ak50 and ak69 and cloned into the *Sal*I-*Sph*I restriction sites of pAK11. This plasmid was designated pAK14 and was introduced into *C. jejuni* NCTC11168 and M1 by natural transformation, generating NCTC11168 Δ*cj0390*b and M1 Δ*CJM1_0368*b.

2.3. Genetic complementation

PCR was used to amplify *cj0390* from NCTC11168 genomic DNA using primers akTAGNF and akTAGNR (Table 1). This allowed an in-frame insertion of a 24 bp *N*-terminal FLAG fusion tag, encoding an eight amino acid epitope tag (DYKDDDDK) [23]. The BamHI-digested PCR product was cloned into BamHI digested, alkaline phosphatase treated pAK14. The resulting plasmid, where *cj0390*, fused to an *N*-terminal FLAG tag, was located in between its native upstream and downstream DNA sequences, was designated pAK28. The

kanamycin resistance gene, *aphA3* [24], was amplified by PCR using primers ak139 and ak55 and cloned into the *Afl*II/*Sal*I sites of pAK28, replacing the *cat* gene. The resultant construct was designated pAK36 and was used to naturally transform *C. jejuni* NCTC11168 Δ*cj0390*b and M1 Δ*CJM1_0368*b. The resulting transformants were designated NCTC11168 *cj0390** or M1 *CJM1_0368**. Southern blot and PCR analysis were performed to confirm allelic replacement for all mutants.

2.4. Motility assays

Motility assays were performed as described by Silverman and Simon [25]. Single colonies were used to inoculate semi-solid (0.4% w/v) MH agar plates. Plates were incubated for 30 hours under microaerophilic conditions. When comparing motilities of wild type and mutant bacteria the diameter of the motility zone was measured for approximately 200 colonies of each strain and the data were analysed using Microsoft Excel and SSPS 11.0 software. The Shapiro-Wilk test of normality was applied to each data set. Since the data was found to be not normally distributed ($P \leq 0.05$ for NCTC11168, $P \leq 0.01$ for NCTC11168 *cj0390**), the Mann-Whitney test was subsequently applied to compare the difference in median motility zone diameter between the samples.

2.5. Phase contrast microscopy

Bacterial cells taken from liquid culture were placed onto Vecta bond treated glass slides SP-1800, (Vector Laboratories) and covered with 22 x 22 mm glass coverslips (Fisher Scientific). Slides were viewed using a Leica DM6000B microscope (Leica Biosystems) using phase contrast settings with oil immersion.

2.6. Transmission electron microscopy

C. jejuni cells, harvested from MH plates, were cultured overnight in MH broth at 42˚C in microaerophilic conditions, shaking at 150 rpm. A copper grid covered with formvar was floated on a droplet of bacterial suspension derived from these overnight cultures. The grid was then washed with 5 droplets of distilled water and finally stained with 2% (w/v) uranyl acetate. Grids were then examined in a CM100 transmission electron microscope (FEI-Philips) at the Cambridge Advanced Imaging Centre, Department of Physiology, Development and Neuroscience, University of Cambridge.

2.7. Preparation and Mass Spectrometry analysis of flagellin

Intact flagella were purified by a modification of the method of Power *et al.* [26]. Briefly, 100 ml of MH broth (pre-warmed to 42^{o}C) was inoculated using an overnight culture at a 1:1,000 dilution. Cultures were incubated for 16 h after which they were pelleted (10,000 x g for 20 min) and re-suspended in 10 mM Tris-HCl, 0.85% (w/v) Tris-NaCl pH 7.4. Samples were placed on ice and homogenised for 2 min using an Ultra-turrax homogeniser, Model T25 S7 (Janke and Kunkel IKA-Labortechnik), in order to shear-off flagella. Cell debris and whole cells were pelleted by centrifugation (10,000 x g at 4^{o}C for 60 min) to pellet flagella. The supernatant was discarded and the pellet washed in 5-10 ml sterile distilled water and ultra-centrifuged again as described. Pelleted flagella were re-suspended in 1-5 ml aliquots and stored at -80^{o}C until needed. Samples were mixed with 0.5 volumes of final sample buffer, boiled for 5 min and subjected to SDS-PAGE. Pre-cast 4-12% NuPAGE Novex Bis-Tris gels (Thermo Fisher Scientific), or resolving and stacking gels prepared as described

in [19], were used in the XCell Surelock Mini-Cell system (Thermo Fisher Scientific), and gels were stained using the Colloidal Blue gel staining kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Gels were destained and analysed by Mass Spectrometry (Centre for proteomic Research, University of Southampton, UK). Protein bands were excised and subjected to in situ tryptic digestion using the method of Shevchenko *et al*. [27]. The resulting peptides were separated by nano-reverse phase liquid chromatography, using a Water C18, 3μm, 100Å (150 mm x 75 μm) column (Waters Biocorporation, USA) and electrosprayed into a Global Ultima quadrupole time-of-flight tandem mass spectrometer (Waters). Operation and data collection was performed using the software MassLynx 4.0 (Waters). All MS/MS spectra were automatically processed using ProteinLynx Global server 2.0 (Waters) and searched against a FASTA format of the NCBI database.

2.8. In vitro *growth kinetics of* C. jejuni

Bacterial colonies were harvested from 2 day old MH plates and used to inoculate MH liquid cultures which were cultured microaerophilically, shaking at 150 rpm, for 16 hours at 42˚C. Optical density (OD) readings were taken at 600 nm and liquid cultures were sub-cultured into flasks containing MH broth, inoculated at 10^4-10^5 colony forming units (CFU) per ml. Flasks were incubated at 42˚C in microaerophilic conditions and bacterial growth was measured at appropriate time intervals by spectrophotometry. In order to determine the number of viable CFU, 100μl aliquots were serially diluted in phosphate buffered saline (PBS) and spread onto MH agar plates at 2-7 h intervals. Following 2 days of incubation at 42˚C in microaerophilic conditions, the number of CFU was counted. Data points are represented as the mean ± the standard deviation calculated from a minimum of triplicate

185 data. Generation time (based on the growth between 5 and 20 h) was calculated as
186 described by Pelczar *et al*. [28].
187

188 ## *2.9. Chicken colonisation experiments*

189 To establish an initial gut flora, day-of-hatch specific-pathogen-free (SPF) Light
190 Sussex chicks were fed with 0.1 ml of *Campylobacter*-free adult gut flora preparations.
191 These preparations were generated by taking 1 g of caecal contents from a 50-week old SPF
192 chicken and using it to inoculate 10 ml of LB broth, which was incubated in static culture for
193 24 h at 37˚C. Birds were fed a vegetable based diet (Special Diet Services). After two weeks,
194 five chickens housed in a single cage, were orally infected with 0.1 ml of a MH broth culture
195 containing $1x10^9$, $1x10^9$, $1x10^{10}$ CFU ml^{-1} of the M1 wild type, M1 Δ*CJM1_0368*b and M1
196 *CJM1_0368**, respectively. Seven days post inoculation (p.i.), chickens were sacrificed and
197 caecal contents were serially diluted in PBS and plated onto blood-free *Campylobacter*
198 selective agar containing CCDA-selective supplement (Oxoid). Plates were incubated in
199 microaerophilic conditions for 48 h and CFU enumerated. The number of *Campylobacter*
200 CFU per gram of caecal content was calculated.
201

202 # 3. Results

203 ## *3.1. Bioinformatics analysis of NCTC11168 Cj0390*

204 NCTC11168 *cj0390* (*pflB*) is predicted to encode a 93.5 kDa protein. PSI-BLAST
205 (http://www.ncbi.nlm.nih.gov/BLASTP) was performed to identify potential protein
206 homologues, in which matches with the highest % identity were found to be against ORFs in
207 other *Campylobacter* species, suggesting that this protein is conserved within the

208 *Campylobacter* genus. Weaker homologues were identified in *Helicobacter* spp.,
209 *Sulfurospirillum* spp., *Wolinella succinogenes* and *Arcobacter butzleri* which are all members
210 of the Campylobacteraceae family within the EpsilonProteobacteria class. The amino acid
211 sequence of NCTC11168 Cj0390 was analysed using the Simple Modular Architectural Tool
212 (SMART) [29] that predicted the presence of four tetratricopeptide repeats (TPRs) (amino
213 acids 145-178, 181-210, 310-343, 499-532), a transmembrane domain (amino acids 90-122),
214 areas of low complexity (amino acids 403-420, 750-763) and a coiled coil region (amino
215 acids 775-796). Proteins with multiple copies of TPRs have been shown to function as
216 scaffolding proteins and coordinate the assembly of proteins into multi-subunit complexes
217 [30].
218
219 ## *3.2. NCTC11168* Δcj0390 *and M1* ΔCJM1_0368 *mutants possess*
220 ## *flagella but are non-motile*
221 Δ*cj0390*b and Δ*CJM1_0368*b generated in *C. jejuni* strains NCTC11168 and M1,
222 respectively, were non-motile on semi-solid agar plates (Fig. 1). When viewed under phase-
223 contrast microscopy, the mutants were non-motile in contrast to the rapid darting motility
224 of *C. jejuni* wild type cells (data not shown). Electron microscopy showed that the
225 NCTC11168 Δ*cj0390*b and M1 Δ*CJM1_0368*b possessed flagella that appeared like the wild
226 type (Fig. 2). To investigate any differences in the glycosylation status of the flagellins,
227 flagella were purified from NCTC11168 wild type and Δ*cj0390*a. Mass spectrometry (MS)
228 (Fig. 3) and isoelectric focusing (IEF) gel analysis was performed on the samples which
229 showed that the flagellins samples had no detectable differences (data not shown). Motility
230 plate assays demonstrated that the complemented NCTC11168 Δ*cj0390*b mutant, *cj0390**

231 was motile. However, the diameter of the motility zone was noted to be reduced in the
232 complemented mutant in comparison to the wild type when approximately 200 colonies of
233 each were analysed (Fig. 4). This suggests that the presence of a FLAG tag at the *N*-terminus
234 may attenuate the function of Cj0390, thereby reducing motility.

235 The *in vitro* growth dynamics in liquid culture of the *C. jejuni* NCTC11168 Δ*cj0390*a
236 mutant was compared with the wild type (Fig. 5). The mutant was found to have a faster net
237 growth rate compared to that of the wild type; the generation time of the wild type was
238 1.44 $\pm$ 0.22 generations per h, compared to 1.09 $\pm$ 0.10 for the mutant, a Student's *t*-test
239 using viable cell counts at 13.5 h (representing the maximal growth rate) was statistically
240 significant ($p \leq 0.05$).

241

242 *3.3.* C. jejuni CJM1_0368 *is essential for the colonisation of 2 week*
243 *old chickens*

244 Chicken colonisation experiments were performed using *C. jejuni* strain M1, a natural
245 poultry isolate which is an efficient coloniser of chickens and is also able to cause disease in
246 humans [31]. Five chickens, housed in a single pen, were inoculated with M1 wild type, M1
247 Δ*CJM1_0368*b or M1 *CJM1_0368**. Seven days p.i., chickens were sacrificed and the caecal
248 contents were spread onto *Campylobacter* selective plates. The viable counts per gram of
249 caecal content revealed that M1 Δ*CJM1_0368*b failed to colonise the chicken caeca (Fig. 6).
250 However, *C. jejuni* M1 wild type and M1 *CJM1_0368** colonised to around 1 x 10^9 CFU g^{-1} of
251 caecal contents.

252

253 4. Discussion

Our findings demonstrate that C. jejuni *pflB*, NCTC11168 *cj0390* and the equivalent gene in M1 (*CJM1_0368*), is required for *C. jejuni* motility. This is supported by a partial restoration of motility of the mutants when the wild type gene is restored *in cis*. Electron microscopy revealed that NCTC11168 *cj0390* deletion mutants possess flagella filaments which appeared visually indistinguishable to those of the wild type. *Campylobacter* flagellin is subject to posttranslational modification. In this study, MS and IEF analyses of flagellin failed to identify any differences in the glycosylation patterns between that of wild type compared to mutant.

We found that the NCTC11168 Δ*cj0390* mutant had a significantly faster growth rate compared to the wild type. This is consistent with previous findings that non-motile mutants grow faster compared to the wild type as demonstrated in *C. jejuni* 81116 *flgS*, *flgR*, *rpoN* and *fliA* mutants [16]. NCTC11168 *cj0390* is predicted to encode a 93.5kDa protein with a transmembrane domain and tetratricopeptide repeats [32] with homologues in other organisms within the EpsilonProteobacteria. Although Cj0390 has no significant homologues in the Enterobacteriaceae, similar phenotypes have been observed in *E. coli* and *Salmonella*, where mutations in the genes encoding the motor and switch proteins (MotA, MotB, FliG, FliM and FliN) result in flagella that assemble but fail to rotate [33-35]. Similar 'paralysed flagella' phenotypes have been reported in *C. jejuni flgP*, *flgQ* and *pflA* deletion mutants [36, 37] although the functions of these proteins are not well defined. Indeed, there appears to be diversity amongst the flagella motor structures between bacteria [14] and proteins such as FlgP, FlgQ, PflA and Cj0390 may represent novel proteins involved with motor function.

During our work, it was shown that a *C. jejuni* 81-176 deletion mutant in the *cj0390* homologue (*CJJ81176_0413*) is non-motile but able to generate flagella. Furthermore a

CJJ81176_0413-GFP fusion protein localised to the poles supporting the idea that this protein is directly involved in flagellar structure or assembly and thus has been named PflB [13]. PflA has been shown to interact with PflB in *in vitro* studies [13]. Our findings, together with these previous studies, suggest that PflB (NCTC11168 Cj0390; M1 CJM1_0368; 81-176 CJJ81176_0413) may be involved in the flagella motor or switch functions or form a structural component of the basal body, either directly or indirectly.

A *C. jejuni* M1 *CJM1_0368* (*pflB*) mutant, failed to colonise the caeca of two week old chickens. The *C. jejuni* wild type and complemented mutants colonised to high levels whilst the mutant was below the limits of detection (100 CFU/g). These results demonstrate the importance of PflB in the colonisation of the chicken caecum and highlight that a functional flagellum is critical for colonisation. This finding supports previous studies where *C. jejuni motA* and *pflA* mutants, shown to be non-motile despite possessing flagella, failed to colonise similar models [18].

In summary, our study further confirms the importance of PflB for functioning of the flagella and demonstrates its requirement for *in vivo* colonisation of the chicken gastrointestinal tract. Further characterisation of this protein will enhance our current understanding of *C. jejuni* flagellar function which is essential for colonisation and pathogenesis.

Acknowledgements

We thank Dr Jeremy Skepper at the at the Cambridge Advanced Imaging Centre, Department of Physiology, Development and Neuroscience, University of Cambridge, for technical assistance with electron microscopy and Prof Diane Newell for *C. jejuni* strain M1.

301 This work was supported by the Department for Environment, Food and Rural Affairs (Defra)
302 Senior Fellowship awarded to D.J.M.

303

304 **Glossary**

305 CFU, Colony Forming Units; GBS, Guillain-Barré Syndrome; IEF, Isoelectric focusing; LB, Luria
306 Bertani; MH, Muller Hinton; MS, Mass Spectrometry; OD, Optical Density; ORF, open
307 reading frame; PBS, Phosphate Buffered saline; p.i., post infection; SPF, Specific-Pathogen-
308 Free; STM, Signature Tagged Mutants; TLR5, Toll-receptor 5; TPR, tetratricopeptide repeats.

309

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403 invasion of eukaryotic cells. Mol Microbiol 14:883-893
404

405 **Table 1. Primer sequences used in this study.**

Primer	Sequence 5′ to 3′
ak1	gggggtaccatggctgaacaagaagatata
ak2	cccggatccttagttgttattaatatcaaa
ak9	tccccccgggggccaaatatagctagg
ak10	tccccccgggaaagcctgttcaacctcaccc
ak47	gggcccggggaattcgcctgtgggtgaagatgaa
ak48	gggcccgggggatccctatactctttaaaaatatttttt
ak50	cctctagagcatgcggagtagaaggtactagca
ak51	ccggatcccttaagctcggcggtgttcctttccaa
ak52	ggggcttaaggtcgaccgctttagttcctaaaggg
ak55	cccggggtcgacctaaaacaattcatccagtaaaat
ak69	ggtctagagtcgacaaatgcttagaaatttctaagcatttttagttaagcttgaag
ak139	cccgggcttaagttgacaatactgataagataatata
akTAGNF	gggggatccatggactacaaggatgacgacgacaaggctgaacaagaagatataatact
akTAGNR	ccccggatccttagttgttattaatatcaaaagtaaaaaattcactct

406

407

408 **Table 2. Bacterial strains and plasmids used in this study**.

Strain or Plasmid	Relevant genotype or description	Source/Reference
Plasmids		
pUC19	Ap^r	[20]
pAK35	Cm^r	[21]
pRY107	Cm^r	[22]
pAK3	Ap^r	This study
pAK4	Ap^r, Cm^r	This study
pAK10	Ap^r	This study
pAK11	Ap^r, Cm^r	This study
pAK14	Ap^r, Cm^r	This study
pAK28	Ap^r, Cm^r	This study
pAK36	Ap^r, Kn^r	This study
E. coli		
DH5α	Subcloning Efficiency™ DH5α™ Competent Cells. F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Life Technologies
C. jejuni		
NCTC11168	Clinical isolate	[30]
M1	Environmental isolate	[29]
NCTC11168 Δ*cj0390*a	Δ*cj0390* (Δ1520bp), Cm^r	This study
NCTC11168 Δ*cj0390*b	Δ*cj0390*, Cm^r	This study
NCTC11168 *cj0390**	(Δ*cj0390*b)::*cj0390,* Kn^r	This study
M1 Δ*CJM1_0368*a	Δ*CJM1_0368* (Δ1520bp), Cm^r	This study
M1 Δ*CJM1_0368*b	Δ*CJM1_0368*, Cm^r	This study
M1 *CJM1_0368**	(Δ*CJM1_0368*b):: Δ*0368*, Kn^r	This study

409

410 Abbreviations for antibiotics: Cm^r, Chloramphenicol; Kn^r, Kanamycin; Ap^r, Ampicillin.

411

Fig. 1. Motility agar plate showing the lack of motility of *C. jejuni* NCTC11168 Δ*cj0390*b. A 0.4% (w/v) semi-solid agar plate inoculated with *C. jejuni* NCTC11168 (left) and NCTC11168 Δ*cj0390* (right). After 24 h incubation at 42˚C in microaerophilic conditions, a zone of motility was observed for the wild type but not for the mutant.

Fig. 2. Representative transmission electron micrographs of A) *C. jejuni* NCTC11168 wild type; B) *C. jejuni* NCTC11168 Δ*cj0390*a (scale bar 1 μm).

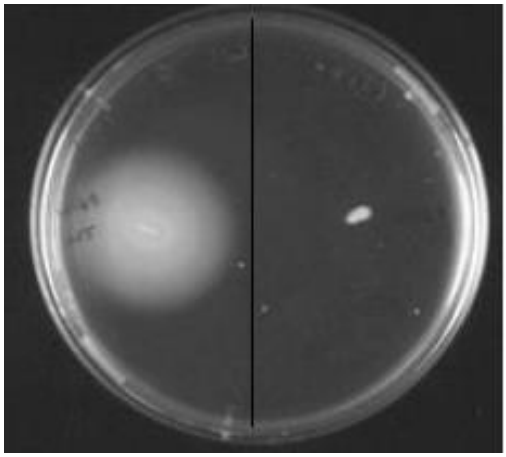
Fig. 3. Representative nanoLC mass spectra of *C. jejuni* NCTC11168 wild type (top) and *C. jejuni* NCTC11168 Δ*cj0390*a sheared flagella. Flagellin bands were excised from a colloidal blue stained gel and subjected to trypsin digestion. Peptides were separated and sprayed into a Global Ultima quadruple time-of-flight tandem MS.

Fig. 4. Motility analysis of *C. jejuni* NCTC11168 wild type and NCTC11168 Δ*cj0390*b complemented mutant, *cj0390**. Motility was assessed on 0.4% (w/v) agar plates after overnight incubation. Median values of the motility zone were 28 mm and 13 mm for the NCTC11168 wild type and *cj0390** strains, respectively; the difference between these medians was statistically significant (Mann-Whitney test, $p \leq 0.01$).

Fig. 5. Net growth rate of *C. jejuni* NCTC11168 wild type and NCTC11168 Δ*cj0390*b**.** The bacteria were cultured in MH broth, shaking at 42˚C in microaerophilic conditions. *C. jejuni* NCTC11168 wild type (red), NCTC11168 Δ*cj0390*b (blue).

435 **Fig. 6.** *Chicken colonisation of C. jejuni M1 wild type, M1 ΔCJM1_0368b and M1*
436 *CJM1_0368*.* Chickens were orally infected with 0.1 ml of a MH broth culture containing 1 x
437 10^9 - 1 x 10^{10} CFU ml^{-1} of the *C. jejuni* strains. Viable counts from serial dilutions of caecal
438 contents of chickens show *CJM1_0368**colonised to similar levels as the wild type but
439 Δ*CJM1_0368* failed to establish an infection (below detectable limits of 100 CFU/g). *C. jejuni*
440 M1 wild type (red), M1 Δ*CJM1_0368*b (blue) and M1 *CJM1_0368** (green).
441

442 **Fig. 1.**



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445 **Fig. 2.**

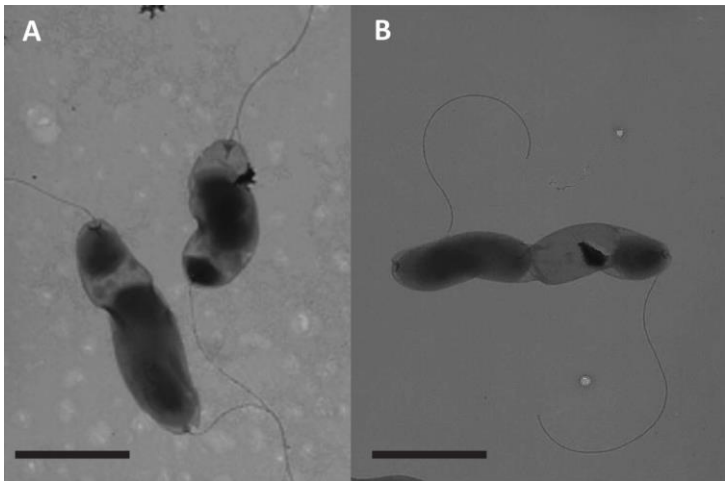
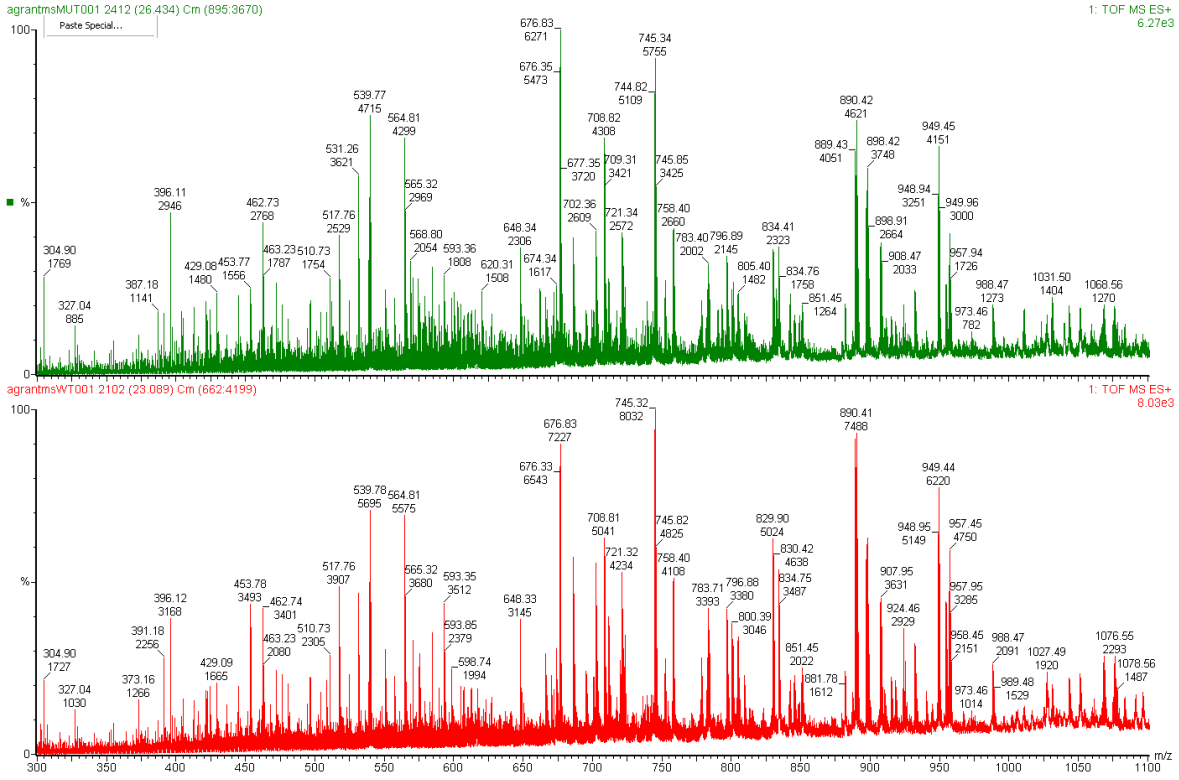


Fig. 3.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m/z
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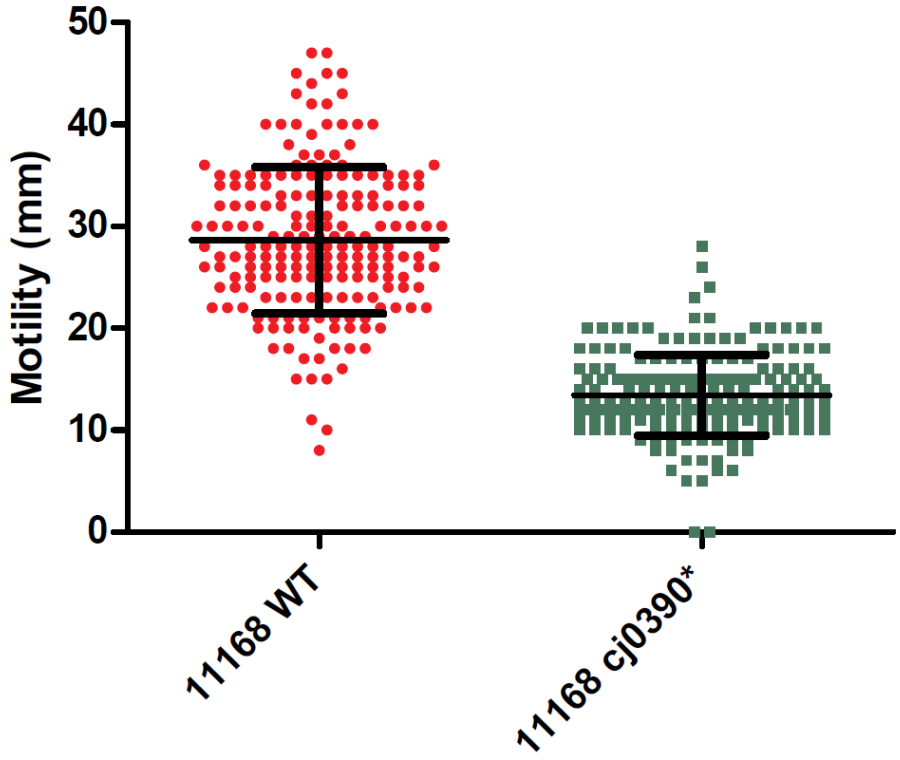
454 **Fig. 4.**



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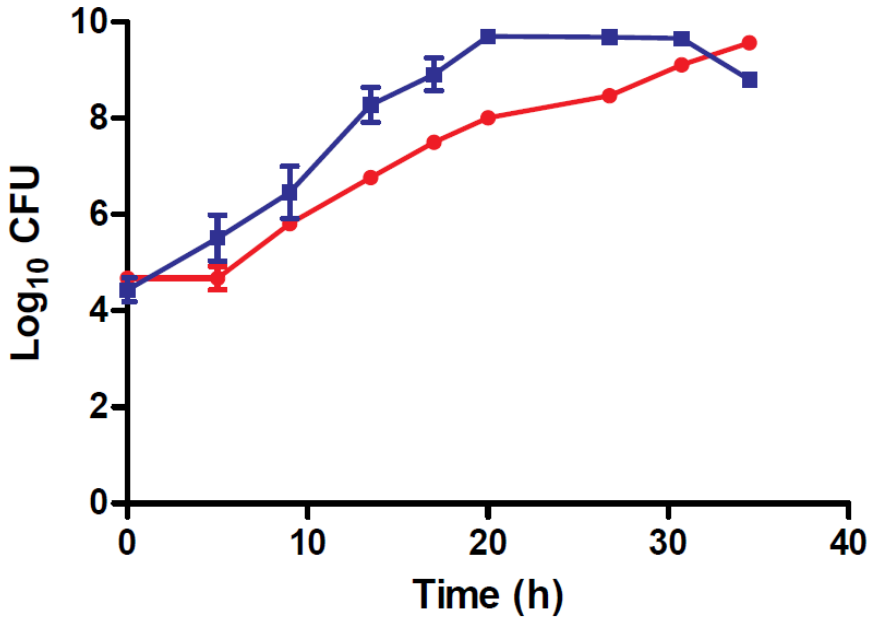
457 **Fig. 5**.



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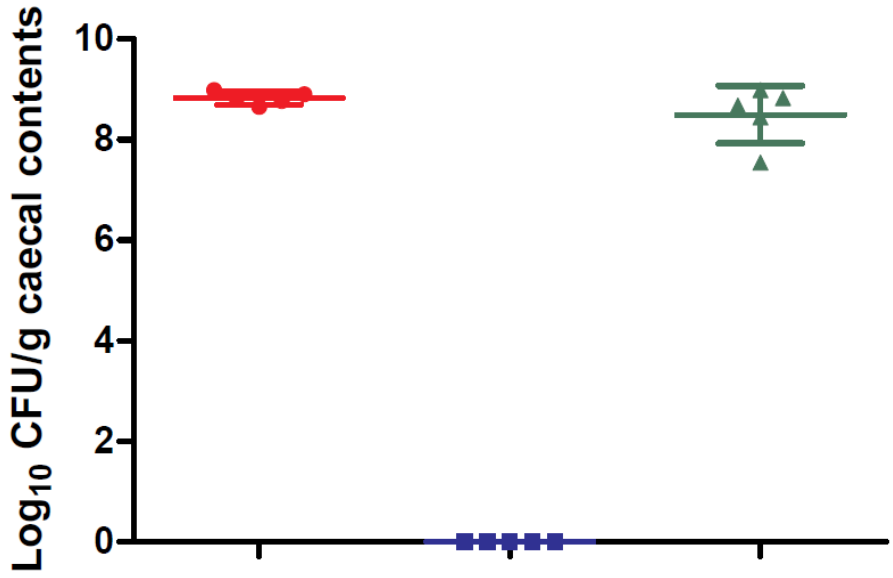
459

460 **Fig. 6.**



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