A quantitative proteomic screen of the *Campylobacter jejuni* flagellar-dependent secretome

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1. Introduction

*Campylobacter jejuni* is the leading cause of bacterial gastroenteritis in the world [1]. Cases of *C. jejuni* infection are most commonly acute and self-limiting in healthy individuals, however a number of complications can occur post-infection. The most serious of these is the development of Guillain-Barré syndrome, an acute demyelinating disease resulting in progressive ascending paralysis [2].

Research investigating *C. jejuni* pathogenesis has identified important roles for flagellum-dependent motility, adhesion/invasion of host epithelial cells and toxin production among others, as factors important for causing human disease [3]. Although these factors are frequently observed among bacterial pathogens, *C. jejuni* appears unlike other enteric pathogens with respect to extracellular protein secretion [4].

It has been proposed that *C. jejuni* utilises its flagellum not only for motility but also to act as a conduit for the secretion of non-flagellar proteins [5]. Previous studies have identified multiple components of the *C. jejuni* flagellum that are required for the export of the *Campylobacter* invasion antigens (Cia), and other non-flagellar proteins, some of which have been implicated in the ability of *C. jejuni* to invade human intestinal cell lines [6–17]. CiaB was the first non-flagellar *C. jejuni* protein proposed to be dependent upon the flagellum for secretion, and is required for efficient invasion of INT-407 cells [9]. CiaB is also suggested to be required for the secretion of at least two other proteins, CiaC and CiaD [7,9,11,12]. CiaC and CiaD secretion requires a minimum flagellar structure containing the hook protein FlgE [7,11,12]. CiaC is necessary for wild type invasion of INT-407 cells [7]. A ciaI mutant of *C. jejuni* F38011 displays reduced survival within INT-407 cells, while a cia mutant of *C. jejuni* 81–176 is reduced in its ability to colonize the chicken intestinal tract [11,12]. Another Cia protein that is dependent on the flagellum for secretion, CiaD, is also required for maximal invasion of INT-407 cells [13,14]. Furthermore, FlcA and FspA also require a minimum flagellar structure for extracellular secretion [15,16]. FlcA, which has high sequence similarity to the major and minor flagellin filament proteins of *C. jejuni*, binds Hep-2 cells, and a *C. jejuni* TGH9011 flaC mutant is reduced in its ability to invade those cells [15]. FspA is readily observed as two isoforms among different *C. jejuni* isolates, and the external addition of FspA2 induces apoptosis of INT-407 cells [16]. Another study has identified a group of proteins dependent on c28 for their production and secretion and hence that are expressed under the same conditions as...
2.2. Isotopic labelling of C. jejuni cultures

Above, and supplemented with 10 mM L-Glutamine. Bacterial strains SILAC data by Western immunoblotting, 1% DMEM plates were comprised of either L-Arginine-HCl (Thermo Fisher Scientific) or L-Arginine 13C6, 15N4 (Thermo Fisher Scientific) or L-Arginine 13C6, 15N4 (Thermo Fisher Scientific) was dissolved in water to create a 2× SILAC DMEM solution. Amounts of either L-Arginine-HCl or L-Arginine 13C6, 15N4 were added to the solution, dissolved, and passed through a 0.22 µm filter. To this, an equal volume of sterile 2% select agar (Sigma) was added to the solution, dissolved, and passed through a 0.22 µm filter.

In this study we have used a combination of SILAC (stable isotope labelling by amino acids in cell culture) and label-free LC-MS (liquid chromatography-mass spectrometry) to investigate the C. jejuni flagellum-dependent secretome. This has enabled a comprehensive screen of the C. jejuni secretome, in an attempt to identify previously undescribed proteins, both flagellar and non-flagellar, being transported via the C. jejuni flagellar type III secretion apparatus (FT3SS). Utilizing C. jejuni strain M1 is a suitable strain for this purpose as it has been documented to colonize both human and avian hosts [19]. Therefore, in using strain M1 we hope to comprehensively assess flagella-dependent proteins, possibly contributing to colonization of chickens and/or the development of human disease.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All wild type strains and defined mutants are described in Table S1. C. jejuni strains were routinely cultured on Brain Heart Infusion (BHI, Oxoid) agar plates supplemented with 5% defibrinated horse blood (Oxoid) and 5 µg/ml trimethoprim (TrM). Strains containing FLAG-tagged proteins were grown in the presence of 50 µg/ml kanamycin (Km). Gene deletion mutants were grown in the presence of 10 µg/ml chloramphenicol (Cm). FLAG-tagged strains also contain gene deletions were grown in the presence of 50 µg/ml Km and 10 µg/ml Cm. Microaerophilic conditions for C. jejuni growth (5% O2, 10% CO2 and 85% N2) were maintained by a MACS VA500 variable atmosphere work station (Don Whitley Scientific).

2.2. Isotopic labelling of C. jejuni cultures

C. jejuni strain M1 and its isotopic flgG mutant were grown on 1% SILAC DMEM plates supplemented with 10 mM l-Glutamine (Sigma) and either l-Aarginine-HCL (Thermo Fisher Scientific) or l-Aarginine 13C6, 15N4 (Thermo Fisher Scientific). Powdered SILAC DMEM (Thermo Fisher Scientific) was dissolved in water to create a 2× SILAC DMEM solution. Amounts of either l-Aarginine-HCL or l-Aarginine 13C6, 15N4 were added to the solution, dissolved, and passed through a 0.22 µm filter. To this, an equal volume of sterile 2% select agar (Sigma) was added and supplemented with 10 mM l-Glutamine. For validation of the SILAC data by Western immunoblotting, 1% DMEM plates were composed of 2× standard DMEM (Millipore) mixed with 2% sterile agar as above, and supplemented with 10 mM l-Glutamine. Bacterial strains were streaked on relevant media from frozen stocks and incubated at 42 °C under microaerophilic conditions for 48 h.

2.3. Preparation of C. jejuni supernatants

For SILAC labelled cultures, once isotopic amino acid incorporation was achieved a previously published protocol for the generation of C. jejuni supernatants [6,12] was utilised, adapted here for use with DMEM. Bacteria were suspended to an OD 600 nm of 0.6 in 20mls SILAC DMEM (Thermo Fisher Scientific) supplemented with a relevant amount of “light” (wild type samples) or “heavy” (mutant samples) l-Aarginine, and 10 mM l-Glutamine. This 20 ml culture was overlaid onto 5 ml 1% SILAC DMEM agar and was incubated statically at 42 °C under microaerophilic conditions for 4 h. Various growth experiments revealed that these conditions were optimal for C. jejuni growth in DMEM (data not shown). For the M1 flgG mutant, chloramphenicol was added at a concentration of 10 µg/ml to both 1% DMEM agar and liquid SILAC DMEM. At the end of the incubation period, OD 600 nm measurements were taken for each culture, and 1 ml of each culture was pelleted for subsequent whole cell protein sample preparation. Each remaining 18 ml culture was centrifuged at 4000 × g for 20 min, the supernatant was transferred to a fresh tube and the centrifugation step was repeated. Supernatants were then passed through a syringe with a 0.22 µm filter to remove any remaining whole bacteria. Following this, 15 ml of each supernatant was transferred to an Amicon Ultra centrifugal filter unit (Millipore) and centrifuged at 4000 g for 30 min. To make supernatant samples for SILAC validation experiments, the above protocol was followed using 1% standard DMEM agar (Gibco) supplemented with 10 mM l-Glutamine. Concentrated supernatant samples were divided into aliquots which were stored at −20 °C for future use.

2.4. LC-MS/MS analysis

The unlabelled, or SILAC labelled samples were reduced with tris(2-carboxyethyl) phosphine (TCEP) then alkylated with iodoacetamide (Sigma) followed by digestion by trypsin (Thermo Fisher Scientific) overnight at 37 °C 0.5 µg (unlabelled samples) or 1.5 µg (SILAC samples) of the digest were submitted for the nano LC-MS/MS analyses on an Ultimate 3000 RSLCnano System coupled to a LTQ Orbitrap Velos hybrid mass spectrometer equipped with a nanospray source. The peptides were first loaded and desalted on a PepMap C18 trap column (100 µm id × 20 mm, 5 µm) then separated on a PepMap C18 analytical column (75 µm id × 500 mm, 2 µm) over a 90 min (unlabelled samples) or 180 min (SILAC labelled samples) linear gradient of 4–32% CH3CN/0.1% formic acid (the HPLC, mass spectrometer and columns were all from Thermo Fisher Scientific). The Orbitrap mass spectrometer was operated in the standard “top 15 or top 10” data-dependent acquisition modes while the preview mode was disabled. The MS full scan was set at m/z 380–1600 with the resolution at 30,000 at m/z 400 and AGC at 1 × 106 with a maximum injection time at 200 msec. The 15, or 10, most abundant multiply-charged precursor ions, with a minimal signal above 3000 counts, were dynamically selected for CID fragmentation (MS/MS) in the ion trap, which had the AGC set at 5000 with the maximum injection time at 100 msec. The dynamic exclusion duration time was set for 60 s with ± 10 ppm exclusion mass width.

The raw files were processed in MaxQuant (Version 1.5.2.8, www.MaxQuant.org) for both protein identification and protein quantification. The C. jejuni M1 protein database was a combination of those downloaded from UniprotKB (www.uniprot.org) of 11,168 (April 2015) and M1 (February 2015). Parameters used were mainly in default values with some modifications: trypsin with maximum 2 missed cleavages, peptide mass tolerance at first search was set at 20 ppm and main search was at 4.5 ppm, MS/MS fragment mass tolerance at 0.50 Da, and top 8 MS/MS peaks per 100 Da and a minimum peptide length of 7 amino acids were required. Fixed modification for Carbamidomethyl and variable modification for Acetyl (Protein N-term), Deamidated (NQ) and Oxidation (M) were used. False discovery rates (FDR) were estimated based on matches to reversed sequences in the concatenated target-decoy database. The maximum FDR at 1% was allowed for proteins and peptide spectrum matches (PSMs). Peptides were assigned to protein groups, a cluster of a leading protein(s) plus additional proteins matching to a subset of the same peptides. For protein quantification, the minimum ratio of two, from 'unique and razor
peptides’ was required, and Re-quantify was enabled but Match between runs was disabled. The protein FDR was set to 0.1%. The MaxQuant output was processed using Perseus (Version 1.5.26 www.MaxQuant.org). Protein groups that are only identified by site, or reverse matches and potential contaminants. Are filtered out. A log2 transformation of SILAC ratio was carried out, rows were filtered to contain a minimum of two values and filtered using a Benjamini-Hochberg FDR test to 0.05. The SILAC data is provided in Table S2, the label-free LC/MS data is provided in Table S3.

2.5. Immunoblotting

Proteins were run on 4–12% SDS polyacrylamide gels, and subsequently transferred to PVDF membranes at 100 V for one hour. All blocking steps were carried out shaking at 30 rpm using 3% fat-free skimmed milk. Primary monoclonal anti-FLAG M2 antibody (Sigma) was used at a concentration of 1:1000. Secondary goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) was used at a concentration of 1:5000. Primary and secondary antibodies were diluted in 3% fat-free skimmed milk. Washing steps were carried out with PBS containing 0.05% Tween 20, and bands were detected using Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

2.6. Generation of defined gene deletion mutants and FLAG-tagged constructs

Construction of gene deletion mutants was carried out by allelic replacement as previously described [20], with each gene of interest being replaced with a chloramphenicol resistance cassette (cat). Primer sequences are provided in Table S4. The cat cassette was amplified from plasmid pCC027 [21] while 5’ and 3’ flanking regions for each gene of interest were amplified from C. jejuni strain M1 genomic DNA. Primers used to amplify the 5’ and 3’ flanking regions also contained overlapping sequence for the cat cassette allowing this to be inserted between the desired 5’ and 3’ sequences by primer-less PCR amplification to create a 5’ flank-cat cassette-3’ flank product. This product was used as a template to amplify the desired sequence for allelic replacement by electroporation, using the forward primer for 5’ flank amplification and the reverse primer for 3’ flank amplification. Electroporation and subsequent natural transformation was carried out as described previously [20], resulting in transformation of C. jejuni strain M1 and the generation of a coupled wild type strain.

FLAG-tagged proteins of interest were generated in C. jejuni M1 and C. jejuni 81–176 by allelic replacement using a kanamycin resistance cassette. Amplification of the kanamycin cassette was carried out using plasmid pRY107 [22] as a template, while flanking regions were amplified using as template genomic DNA from either C. jejuni M1 or C. jejuni 81–176, genomic DNA. For each FLAG-tagged construct, a 5’ flanking region was amplified consisting of 400 bp upstream and an ORF of interest with the FLAGE sequence incorporated immediately before the stop codon. The reverse primer for the 5’ flanking region also contained overlapping sequence with the kanamycin resistance cassette. The 3’ flanking region consisted of 400 bp downstream of the ORF of interest with the forward primer containing an overlapping sequence with the kanamycin resistance cassette. As above for gene deletion, primer-less PCR amplification was carried out to combine a 5’ flank-kanamycin cassette-3’ flank product. This product, containing the kanamycin resistance cassette, was then used to PCR amplify the desired sequence for allelic replacement, by using the forward primer for the 5’ flank amplification and the reverse primer from the 3’ flank amplification. Electroporation and natural transformation for native FLAG-tag incorporation was carried out as described for the creation of defined gene deletions [20].

2.7. Motility assay

C. jejuni was grown on BHI blood agar plates for ~48 h then re-plated on BHI blood plates for ~16 h. Suspensions of C. jejuni scraped from plates into BHI were diluted to OD600nm of ~0.5 and were used to test motility plates comprised of BHI broth containing 0.4% select agar (Sigma). Motility plates were incubated for ~16 h, following which the diameter of the zone of motility was measured (n = 3).

2.8. Culture of CACO-2 cells

CACO-2 cell lines were purchased from the ATCC (CC-L244, HTB-37). Cells were grown using DMEM (Gibco) supplemented with 10% FBS and 1% non-essential amino acids. Cells were routinely grown in 75 cm2 tissue culture flasks and incubated at 37 °C with 5% CO2 in a humidified atmosphere.

2.9. CACO-2 cell infection assays

CACO-2 cells were seeded at 2 × 105 cells on 12 well plates (Greiner) until confluency was observed. CACO-2 cells were infected with different C. jejuni strains at a multiplicity of infection of 100. To assay adherence/invasion, infected cells were incubated with 5% CO2 in a humidified atmosphere for 2 h. At this point non-adherent bacteria were removed, subjected to 10-fold serial dilutions and plated on BHI blood agar plates with 5 μg/ml TrM. Wells were washed three times with PBS, and cells were lysed with 0.1% Triton-X-100 in PBS for 15 min. lysed cells were subjected to 10-fold serial dilutions and plated on BHI blood agar plates with 5 μg/ml TrM. To determine the number of internalized bacteria, infected CACO-2 cells were incubated at 37 °C with 5% CO2 in a humidified atmosphere. After 2 h, the medium overlaying the infected cells was changed to complete DMEM containing 250 μg/ml gentamycin sulphate and infected cells were incubated with 5% CO2 in a humidified atmosphere for a further 2 h. Cells were then washed three times with PBS and lysed with 0.1% Triton-X-100 in PBS for 15 min. Serial dilutions of the cell lysates were carried out and plated on BHI blood agar plates with 5 μg/ml TrM. Dilutions of mutant C. jejuni strains were plated on BHI blood agar plates containing 10 μg/ml chloramphenicol. All plates were incubated for 48 h under microaerophilic conditions at 42 °C before colony counting took place. For both total association and invasion experiments, the percentage of C. jejuni interacting with CACO-2 cells was calculated as a percentage of the non-adherent fraction, to account for potentially different survival profiles of different strains in DMEM (n = 3).

3. Results

3.1. The C. jejuni M1 FT3SS-dependent secretome, as defined by quantitative proteomics

We designed a quantitative proteomics experiment utilizing SILAC to screen proteins whose secretion from C. jejuni strain M1 may be mediated by the FT3SS (Fig. 1). To inhibit flagellar secretion an isogenic M1 flgG mutant was generated. C. jejuni strains with a disrupted flgG gene are inhibited in their secretion of several proteins via the FT3SS [6]. The vast majority of proteins identified by SILAC analysis were similarly abundant in WT and flgG mutants [23]. Proteins possessing H/L ratios lower than −2 (H = mutant labelled with “heavy” l-arginine, L = WT labelled with “light” l-arginine) from SILAC analysis are shown in Table 1. Unsurprisingly, these comprised proteins associated with flagellar gene regulation or structure (FlgM, FlaG), but also FlaC, CiaC, CJM1_1572 and CJM1_0369. A number of proteins were found at an increased abundance within flgG supernatants. Proteins possessing the highest H/L ratios largely represented factors that accommodate correct
flagellar assembly (Fig. 2). To increase the proteome coverage, and also account for potential incomplete incorporation of “heavy” or “light” arginine in the mutant or WT, proteomic analysis of unlabelled M1 flgG and WT supernatants was also performed. Proteins detected within the supernatant of M1 WT but not within flgG supernatants are shown in Table 2. From the SILAC and unlabelled LC/MS data, 5 proteins were chosen for further investigation (CiaI, FlaC, FspA, CJM1_0791 and CJM1_0395). These proteins represented non-flagellar proteins, none of which has been characterized in strain M1, that were present at contrasting abundance within WT and flgG supernatants.

3.2. FLAG-tagging of protein validates MS datasets

A number of the proteins selected for further investigation were FLAG-tagged and assessed for their presence in supernatants obtained from M1 WT and flgG strains. As shown in Fig. 3, CiaI and FlaC were present in the concentrated supernatants from both M1 WT and flgG, although at a very reduced level from the flgG mutant. CJM1_0791 and CJM1_0395 were present only within M1 WT supernatants, confirming the data obtained by label-free LC/MS. Analysis of CysM was included for FLAG-tagging as it has previously been used as a marker for cell lysis [13]. Surprisingly CysM was readily detected within supernatant samples isolated from both M1 WT and flgG within the SILAC data.

3.3. Defined gene deletion mutants alter the interaction of C. jejuni with CACO-2 cells

Individual isogenic deletion mutants of M1 were generated that lack ciaI, flaC, fspA, CJM1_0791 and CJM1_0395 were generated. The abilities of these mutants to interact with CACO-2 cells were measured. Rates of growth and motility for each of the mutants were consistent with

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**Table 1**

| Ratio H/L normalized | Description |
|----------------------|-------------|
| −5.22125             | FlaG        |
| −4.90379             | CiaC        |
| −4.76184             | FlgF        |
| −3.93479             | Flgi        |
| −3.83989             | CJM1_1572   |
| −2.514               | FliD        |
| −2.23358             | CJM1_0369   |
| −2.11747             | FlaC        |

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**Fig. 1.** Overview of M1 secretome analysis. After isotopic incorporation of C. jejuni M1 WT and flgG strains with either “light” or “heavy” arginine, respectively (H = mutant labelled with “heavy” L-arginine, L = WT labelled with “light” arginine), bacterial cells were pelleted and supernatants were filtered, pooled and concentrated. After digestion, tryptic peptides were analysed by LC-MS/MS. Proteins secreted via the flagellum are characterized by enrichment of peptides containing “light” isotopes.

**Fig. 2.** Mean H/L ratios of proteins detected in culture supernatants from four biological replicates. Low H/L ratios represent proteins found at a lower abundance within flgG supernatants while high H/L are proteins found at increased abundance within flgG supernatants.
Table 2

Proteins identified within M1 WT supernatants that were absent in M1 flgG mutant supernatants as detected by standard LC/MS.

| Razor + unique peptides WT | Razor + unique peptides flgG | Q-value | Description |
|---------------------------|-----------------------------|---------|------------|
| 9                         | 0                           | 0       | Uncharacterized protein CJM1_1572 |
| 8                         | 0                           | 0       | Uncharacterized protein CJM1_0791 |
| 7                         | 0                           | 0       | Cia |
| 6                         | 0                           | 0       | FspA |
| 4                         | 0                           | 0       | Uncharacterized protein CJM1_0395 |
| 3                         | 0                           | 0       | Uncharacterized protein CJM1_0821 |
| 3                         | 0                           | 0       | Uncharacterized protein CJM1_1598 |
| 2                         | 0                           | 0       | PurD |
| 2                         | 0                           | 0       | MutS |
| 2                         | 0                           | 0       | RapT |
| 2                         | 0                           | 0       | CmeE |
| 2                         | 0                           | 0       | IspH |
| 2                         | 0                           | 0       | FlgG |
| 2                         | 0                           | 0       | PirZ |
| 2                         | 0                           | 0       | PseA |
| 2                         | 0                           | 0       | FIE |
| 2                         | 0                           | 0       | FlgB |

3.4 C. jejuni strain 81–176 CJM1_0791 and CJM1_0395 genes are dependent on the flagellum for translocation to the supernatant

To investigate whether the absence of CJM1_0791 (CJJ81176_0835) and CJM1_0395 (CJJ81776_0441) within M1 flgG supernatants was strain-specific, these proteins were FLAG-tagged in C. jejuni 81–176 WT and 81–176 flgG backgrounds. The Cia and CysM protein equivalents were also FLAG-tagged. As shown in Fig. 5, CJJ81176_0441 (equivalent to CJM1_0395) was absent from the 81–176 flgG supernatant, consistent with data from M1. A very low level of CJJ81176_0835 (equivalent to CJM1_0791) was detected within the flgG supernatant, although there was a much higher abundance of the protein within the 81–176 WT supernatant. Furthermore, Cia was present at a much higher level within the WT supernatant although still detectable within the flgG mutant supernatant, while CysM was readily detectable within the supernatants from both strains.

4. Discussion

The mechanisms by which C. jejuni invades human intestinal cell lines without a dedicated system for the secretion of effector proteins associated with virulence remains unknown, and is among the most important subjects for study regarding C. jejuni pathogenesis. Previous studies have made good progress in identifying the C. jejuni flagellum as an organelle that mediates extracellular secretion of non-flagellar proteins. Some of these secreted proteins have been proposed to contribute to the invasion of human cell lines by C. jejuni [5–15]. In this study we have used a combination of SILAC and label-free LC/MS to screen C. jejuni secretomes obtained from WT M1 and flagellum-deficient strains.

SILAC analysis measuring the relative abundances of proteins from M1 WT and flgG supernatants revealed that most proteins were present at a relatively equal abundance within supernatants from the WT and flgG mutant (Fig. 2). Similar observations have been made for SILAC secretome studies using S. enterica and E. coli in the past [23,24]. Of the proteins that scored the lowest H/L ratios, the presence of Cia and FlaC adds further support to existing evidence for their proposed utilisation of the flagellum as a mechanism of extracellular secretion [11,15]. It was also observed that Cia was secreted from M1 WT when analysed by label-free LC-MS. The role of the flagellum in Cia and Flac secretion was further confirmed by FLAG-tagged Cia and Flac being present at reduced levels (as detected by Western blotting using an antibody against the FLAG-tag) in flgC mutant supernatants (Fig. 3 and Fig. 5). The reduced abundance of Flac in flgG supernatants is in agreement with the M1 SILAC data; this has not been observed in previous studies [11,15]. Infection assays revealed no apparent influence of Cia on the ability of M1 to adhere to or invade CACO-2 cells. Disruption of flac appeared to reduce levels of both M1 adherence and invasion of CACO-2 cells (Fig. 4). For Cia this is at odds with what has been described regarding invasion using ciaI mutants in strains 81–176 and F38011 [11,12]. It may be that either M1 ciaI does not display the same phenotype as in 81–176 or F38011, or that a reduced interaction for any C. jejuni ciaI mutant does not occur for CACO-2 cells as it does for T84 cells. Previously it has been described that flaC disruption reduces Hep-2 cell invasion by C. jejuni TGH9011 but does not alter adherence [15], as it does in M1. Further work is required to establish the function of Flac across multiple C. jejuni strains, and its role during C. jejuni pathogenesis. It is also notable that neither CiaC (CJM1_1224) nor CiaD (CJM1_0764) were detected in the SILAC data set. However, both proteins were detected in both WT and flgG supernatants in the label-free LC/MS data set. Detection of proteins within the LC/MS but not the SILAC dataset may represent an incomplete incorporation of exogenous arginine labelling, as the external addition of “heavy” or “light” arginine is the only difference between media used for the SILAC and label-free LC/MS experiments and highlights the importance of unlabelled LC/MS to account for this possibility. This may also account for the lack of CiaD detection within the SILAC dataset as the protein contains a single arginine. Previous work has shown that Cia expression and secretion is increased in the presence of external stimuli [25,26]. An excellent future use of SILAC would be to compare supernatants isolated from bacteria in the presence or absence of a stimulus such as bile salts. This would enable a quantitative measurement of the effect of stimuli on Cia secretion, and may facilitate the identification of further proteins, part of the Cia family or otherwise, which contribute to C. jejuni human tissue culture cell interaction.
CJM1_0791 and CJM1_0395 were chosen for further investigation. Immunoblotting of each of these FLAG-tagged proteins reflected the levels observed in either the SILAC or label-free LC/MS data sets. The dependence of CJM1_0791 and CJM1_0395 on the flagellum for their presence in the supernatant is to our knowledge the first time that this has been reported. CJM1_0791 is a putative periplasmic lipoprotein. Disruption of CJM1_0791 led to a reduction in both rates of adhesion and invasion (Fig. 4). The decreased adherence of the CJM1_0791 mutant, its status as a putative lipoprotein, and its relatively large molecular weight (48.9 kDa), make it possible that its absence within flagG supernatants is an indirect effect of knocking out the flagellum. The disruption of a large trans-membrane bound organelle, such as the flagellum, could feasibly lead to membrane structural alteration and a reduction in the presence of other proteins predicted to be membrane bound such as CJM1_0791. Further studies will be necessary to observe whether the protein actually passes through the flagellum, or to identify what secondary effects resulting from flagellar disruption result in its absence from the supernatant. In fact, it was noted that a small number of proteins, which one would not expect to be secreted, were consistently reduced in their abundance within the SILAC dataset from the M1 flagG supernatants (Fig. 2). Similar findings have occurred when using SILAC to study the secretomes of other bacteria, such as the ribosomal protein L21 being present in the SPI-2 (Salmonella Pathogenicity Island 2) dependent secretome of S. enterica [23]. The presence of CysM within both WT and flagG supernatants was a surprising observation considering its use as a marker for cell lysis in the past [13]. It is clear from the data shown here that CysM is found within M1 and 81–176 supernatants, although the reasoning for this is unclear. Future studies will be needed to determine whether CysM is found within the supernatants of more C. jejuni strains, as has been observed here for M1 and 81–176. CJM1_0395 (26 kDa) is a member of the SprA-related superfamily of zinc metalloproteases, and represents a good candidate as a novel non-flagellar protein secreted via the FT3SS. Another zinc metalloprotease, NleC, present in E. coli and S. enterica and exported via the T3SS also does not have a large effect on rates of epithelial cell invasion as observed for a CJM1_0395 mutant, but impairs an NF-kB mediated inflammatory response during infection [27–29]. Although there is no sequence similarity between the two proteins, future work will be required to address whether CJM1_0395 is delivered to host cells, and whether it may contribute to aspects of C. jejuni disease other than a direct influence on rates of adhesion/invasion of in vitro grown tissue culture cells.

In conclusion, in this study we have applied quantitative proteomics to study the C. jejuni flagellum-dependent secretome quantitatively for the first time. The combination of SILAC and unlabelled proteomics has led to the identification of two proteins, CJM1_0791 and CJM1_0395, which appear dependent on a WT flagellum for their presence within C. jejuni supernatant, representing putative effector proteins that regulate infection. We have also further defined the influence of the flagellum on Cial and FlaC secretion for C. jejuni strain M1, and identified novel genes that alter the ability of M1 to interact with CACO-2 cells. Future work combining the use of existing and developing proteomic technologies will allow for a significantly more in-depth assessment of C. jejuni biology and host-pathogen interactions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2016.11.009.

Conflict of interest

The authors declare that there is no conflict of interest.
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