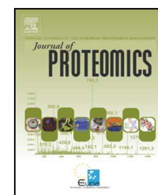
Journal of Proteomics 152 (2017) 181–187

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



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

Eoin Scanlan [a,1], Lu Yu [b], Duncan Maskell [a], Jyoti Choudhary [b], Andrew Grant [a,*]

[a] Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, United Kingdom
[b] Proteomic Mass Spectrometry, Wellcome Trust Sanger Institute, Hinxton CB10 1SA, United Kingdom

ARTICLE INFO

Article history:
Received 12 July 2016
Received in revised form 11 November 2016
Accepted 14 November 2016
Available online 16 November 2016

Keywords:
Campylobacter jejuni
Secretome
Type III secretion system
Flagella
SILAC
Mass spec

ABSTRACT

Campylobacter jejuni is the leading cause of bacterial gastroenteritis in the world. A number of factors are believed to contribute to the ability of *C. jejuni* to cause disease within the human host including the secretion of non-flagellar proteins *via* the flagellar type III secretion system (FT3SS). Here for the first time we have utilised quantitative proteomics using stable isotope labelling by amino acids in cell culture (SILAC), and label-free liquid chromatography-mass spectrometry (LC/MS), to compare supernatant samples from *C. jejuni* M1 wild type and flagella-deficient (*flgG* mutant) strains to identify putative novel proteins secreted *via* the FT3SS. Genes encoding proteins that were candidates for flagellar secretion, derived from the LC/MS and SILAC datasets, were deleted. Infection of human CACO-2 tissue culture cells using these mutants resulted in the identification of novel genes required for interactions with these cells. This work has shown for the first time that both CJM1_0791 and CJM1_0395 are dependent on the flagellum for their presence in supernatants from *C. jejuni* stains M1 and 81–176.

Biological significance: This study provides the most complete description of the *Campylobac er jejuni* secretome to date. SILAC and label-free proteomics comparing mutants with or without flagella have resulted in the identification of two *C. jejuni* proteins that are dependent on flagella for their export from the bacterial cell.

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1. Introduction

Campylobacter jejuni is the leading cause of foodborne 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 Guillian-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 CiaI [7,9,11,12]. CiaC and CiaI 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 *ciaI* 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, FlaC and FspA also require a minimum flagellar structure for extracellular secretion [15,16]. FlaC, which has high sequence similarity to the major and minor flagellin filaments 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 σ^{28} for their production and secretion and hence that are expressed under the same conditions as

* Corresponding author at: Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, United Kingdom.
E-mail address: ajg60@cam.ac.uk (A. Grant).
[1] Current address: San Francisco Veterans Affairs Medical Center and the University of California, San Francisco, USA.

http://dx.doi.org/10.1016/j.jprot.2016.11.009
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flaA, resulting in these being annotated as the Feds proteins (flagellar co-expressed determinants). This group of four proteins is required for colonization of chickens, with FedA also important for invasion of human-derived T84 cells [12].

Although multiple *C. jejuni* proteins are dependent on the flagellum for their secretion, a possible mechanism by which it might interact with host cells has yet to be described. Moreover, the ability of a *C. jejuni* strain 81–176 *ciaB* mutant to invade T84 cells is not statistically different from the wild type [17]. Much of the literature describing non-flagellar protein secretion *via* the *C. jejuni* flagellum has documented proteins secreted to the extracellular environment. The biological relevance of this for effector-like proteins is unclear, as they are likely to be subjected to degradation by host proteases. There has also been no identification of a conserved amino acid sequence present among non-flagellar *C. jejuni* proteins that might act as a flagellar secretion signal, as has been described for the *Yersinia enterocolitica* protein YplA [18].

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-dependant 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* stain 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 containing gene deletions were grown in the presence of 50 μg/ml Km and 10 μg/ml Cm. Microaerophilic conditions for *C. jejuni* growth (5% O_2, 10% CO_2 and 85% N_2) 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 isogenic *flgG* mutant were grown on 1% SILAC DMEM plates supplemented with 10 mM L-Glutamine (Sigma) and either L-Arginine-HCL (Thermo Fisher Scientific) or L-Arginine $^{13}C_6$, $^{15}N_4$ (Thermo Fisher Scientific). Powdered SILAC DMEM (Thermo Fisher Scientific) was dissolved in water to create a 2× SILAC DMEM solution. Amounts of either L-Arginine-HCL or L-Arginine $^{13}C_6$, $^{15}N_4$ 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_{600nm} 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-Arginine, 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_{600nm} 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% CH_3CN/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-dependant 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 1×10^6 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 cleavage sites, 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

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