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Proteomic and genomic analysis reveals novel *Campylobacter jejuni* outer membrane proteins and potential heterogeneity

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ABSTRACT

Gram-negative bacterial outer membrane proteins play important roles in the interaction of bacteria with their environment including nutrient acquisition, adhesion and invasion, and antibiotic resistance. In this study we identified 47 proteins within the Sarkosyl-insoluble fraction of *Campylobacter jejuni* 81-176, using LC–ESI-MS/MS. Comparative analysis of outer membrane protein sequences was visualised to reveal protein distribution within a panel of *Campylobacter* spp., identifying several *C. jejuni*-specific proteins. Smith–Waterman analyses of *C. jejuni* homologues revealed high sequence conservation amongst a number of hypothetical proteins, sequence heterogeneity of other proteins and several proteins which are absent in a proportion of strains.

1. Introduction

Campylobacter spp. are the commonest cause of food-borne disease worldwide accounting for 2.4 million cases per year in the US (www.cdc.gov/foodnet) with *Campylobacter jejuni* and *C. coli* responsible for the majority of infections. In the developing world these organisms are the leading cause of gastrointestinal infection in children under 2 years of age [1]. Additionally, post-infection sequelae may arise including Guillain–Barré syndrome (GBS) and other debilitating neurological disorders [2,3]. Despite a significant worldwide effort, mechanisms of disease and immunity remain poorly understood. Gram-negative bacterial outer membrane (OM) proteins represent a group of factors which play important roles in the interaction of bacteria with their environment. These include porins and nutrient uptake systems, iron acquisition proteins,

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virulence factors, proteins involved in antibiotic resistance and other proteins required for survival within the hostile *in vivo* environment.

To date, functional roles have been ascribed to only a dozen or so *C. jejuni* OM proteins. A more comprehensive characterisation of this important group of proteins, even in prototypic strains, has yet to be reported. Several *C. jejuni* surface proteins have been observed to play roles in adhesion. Three of these, CadF and FlpA, and PEB1 have been shown to play a role in the colonisation of broiler chicks [4,5]. Additionally, JlpA, CapA and PorA appear to mediate binding to epithelial cells in culture [6–8]. Another important role is the transportation of small molecules across the bacterial cell membrane. *C. jejuni* lacks the phosphofructokinase protein and cannot therefore metabolise glucose although recently some strains were shown to use fucose as a substrate for growth [9,10]. Instead, the bacterium utilises amino acids as a source of carbon and energy; the surface proteins PEB1, and CjaA are components of ABC transporters with roles in aspartate/glutamate, and cysteine uptake respectively [11,12]. OM efflux systems are also important: CmeC and CmeD are OM components of two functionally characterised multi-drug efflux pumps, which have roles in antimicrobial and bile resistance [13,14]. Omp50 and PorA have been characterised as porins and the former was recently shown to play a role in phosphotyrosine network regulation [15–17]. *Campylobacter* spp. also possess a number of iron acquisition systems, components of which reside in the OM including the Fe^{3+} enterobactin binding proteins CfrA and CfrB [18–20] and the heme transporter ChuA, [21]. Flagella have a variety of proposed roles in *C. jejuni* virulence and protein components which anchor flagella to the membrane (FlgH) are located in the OM [22]. OM proteins may also elicit pathological host responses; the surface-exposed lipoprotein JlpA is believed to trigger signalling events, which lead to inflammation [23].

Interrogation of the *C. jejuni* genome has revealed that a number of protein complexes and chaperones, which are essential for membrane biogenesis in many well characterised Gram-negative bacteria, are either divergent or absent altogether within *C. jejuni* genomes [24]. The β-barrel assembly machinery is known as the Bam complex [25]. The closest homologue of the OM localised component, BamA (formerly known as YaeT), is divergent in *C. jejuni* and, to the best of our knowledge, homologues of BamBCDE, are not found at all within *Epsilonproteobacteria* genomes. LolB of the Lol (lipoprotein localisation) complex, which is responsible for sorting lipoproteins [26] is absent in *Epsilonproteobacteria*, although homologues of other proteins within this complex are present in *C. jejuni* genomes. *C. jejuni* also varies from the majority of other Gram-negative bacteria in that it lacks the O-antigen of LPS. Often referred to as LOS (lipooligosacharide), this is perhaps a reflection of the divergence of the Lpt (*LPS transport*) proteins which are required for insertion of LPS into the outer membrane. This suggests that alternative mechanisms of maintaining membrane integrity and biogenesis have yet to be revealed within *C. jejuni* and related bacteria. Existing evidence suggests that distinct mechanisms for OM biogenesis exist outside the *Gammaproteobacteria* group of bacteria [27,28]. The divergence in this cellular machinery for protein sorting also suggests that conventional *in silico* protein localisation tools may not be reliable for this particular group of bacteria and therefore localisation of these proteins by experimental methods is particularly pertinent.

Approximately one third of *C. jejuni* predicted open reading frames (ORFs) code for proteins of unknown function and it is unlikely that the current list of characterised OM proteins is complete. Therefore, a thorough analysis of expressed OM proteins is essential for identifying factors important to and possibly novel to *C. jejuni* pathogenesis. We performed proteomic analysis of the OM of *C. jejuni* subsp. *jejuni* 81-176, a widely employed strain which causes experimentally reproducible clinical disease [29]. A variety of methods have been used previously for membrane protein enrichment although the fractions of extracted proteins vary in composition. The most comprehensive proteome analysis of *C. jejuni* to date focussed on the entire membrane compartment, *i.e.* periplasm and both inner and outer membranes [30]. *Campylobacter* OM appears to be closely associated with the inner membrane and as a result is more challenging to purify [31]. Recently, Hobb et al. [32] reported that *N*-lauroylsarcosine (Sarkosyl) treatment of *C. jejuni* cells was the most successful method of isolating specifically OM proteins. Furthermore, Sarkosyl enrichment is often used to predict localisation of *Campylobacter* proteins [15,33,34].

The low solubility of OM proteins renders them incompatible with the majority of proteomic techniques hence gel-based proteomics offers a convenient method for their analysis as issues associated with hydrophobicity are circumvented by ionic detergent (sodium dodecyl sulfate) solubilisation and subsequent in-gel tryptic digestion [35,36]. In this study, a rapid shotgun proteomics-based approach was used to catalogue the protein complement of the *C. jejuni* OM fraction. This methodology, comprising SDS-PAGE, one-dimensional monolithic column liquid chromatography, electrospray ionisation (ESI) and fast MS/MS scanning, is colloquially termed "sawn-off shotgun proteomic analysis" (SOSPA). This approach enables the analysis of membrane-associated and other hydrophobic proteins whilst simultaneously combining rapidity with breadth of coverage. Bioinformatic approaches were deployed to survey the resulting SOSPA-generated data to identify homologous proteins amongst bacterial, *Epsilonproteobacteria* and, particularly, *Campylobacter* genomic sequences.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

C. jejuni 81-176 (pVir+) is a well characterised strain, isolated from contaminated milk [37]. Bacteria were grown at 37 °C in a variable atmosphere incubator (Don Whitley Scientific, Shipley, UK) in an atmosphere of 6% hydrogen, 5% carbon dioxide, 5% oxygen, and 84% nitrogen. Bacteria were cultured for 48 h on *Campylobacter* selective agar (Skirrow) plates (E&O Laboratories, Bonnybridge, UK) then resuspended in 20 ml high glucose Dulbecco's modified Eagle's medium (cat. 11960, Invitrogen, Paisley, UK), supplemented with 20 mM L-glutamic acid and 0.00125% iron ascorbate (Sigma, Dorset, UK) [38] at an Abs_{600} of