Microbial Pathogenesis 104 (2017) 202–211

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

Identification and initial characterisation of a protein involved in *Campylobacter jejuni* cell shape

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ARTICLE INFO

Article history:
Received 20 September 2016
Received in revised form
11 January 2017
Accepted 24 January 2017
Available online 25 January 2017

Keywords:
Campylobacter jejuni
Cell shape

ABSTRACT

Campylobacter jejuni is the leading cause of bacterial food borne illness. While helical cell shape is considered important for *C. jejuni* pathogenesis, this bacterium is capable of adopting other morphologies. To better understand how helical-shaped *C. jejuni* maintain their shape and thus any associated colonisation, pathogenicity or other advantage, it is first important to identify the genes and proteins involved. So far, two peptidoglycan modifying enzymes Pgp1 and Pgp2 have been shown to be required for *C. jejuni* helical cell shape. We performed a visual screen of ~2000 transposon mutants of *C. jejuni* for cell shape mutants. Whole genome sequence data of the mutants with altered cell shape, directed mutants, wild type stocks and isolated helical and rod-shaped 'wild type' *C. jejuni*, identified a number of different mutations in *pgp1* and *pgp2*, which result in a change in helical to rod bacterial cell shape. We also identified an isolate with a loss of curvature. In this study, we have identified the genomic change in this isolate, and found that targeted deletion of the gene with the change resulted in bacteria with loss of curvature. Helical cell shape was restored by supplying the gene *in trans*. We examined the effect of loss of the gene on bacterial motility, adhesion and invasion of tissue culture cells and chicken colonisation, as well as the effect on the muropeptide profile of the peptidoglycan sacculus. Our work identifies another factor involved in helical cell shape.

1. Introduction

Infection by *Campylobacter* spp, especially *Campylobacter jejuni*, is considered to be the most prevalent cause of bacterial diarrhoeal disease worldwide [1]. The bacterium is found in the gastrointestinal tract of healthy animals, especially chickens, destined for human consumption. The helical shape of *C. jejuni* is believed to be important for the bacteria to colonise chickens and during infection, to move through the mucus layer of the gastrointestinal tract and to 'corkscrew' into the cells of a human (or other animal) host.

There is limited understanding of how *C. jejuni* adopts a helical morphology. One study identified a mutation in *flhB* that affected flagella formation and apparently correlated with *C. jejuni* becoming rod-shaped [2], but mutations at other sites in the same flagellar gene resulted in bacteria that remained helical. A mutant in *cj1564* (transducer-like protein 3, Tlrp3) has many altered phenotypic characteristics including loss of curvature, but the mechanism for the change in shape is not clear [3]. Occasionally, laboratory strains of *C. jejuni* lose cell curvature and become rod

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http://dx.doi.org/10.1016/j.micpath.2017.01.042

shaped [4]. *C. jejuni* can also undergo a transition from helical cells to rod shaped or coccoid forms in older cultures, and under conditions of stress. It is not clear whether *C. jejuni* can move back and forth between different conformational states during growth. The only genes known to be involved in determination of the helical cell shape of *C. jejuni* are *pgp1* and *pgp2* [5–7], and their protein products are peptidoglycan (PG) peptidases that are important for PG modification [5,6].

The bacterial cell wall is important for providing both rigidity and shape to cells and is composed of layers of PG, or murein, which forms the murein sacculus [8]. In Gram-negative bacteria, such as *C. jejuni*, the murein sacculus is very thin and lies in the periplasm between the inner and outer membranes. PG is a web of glycan polymers joined by peptide side chains, which are either directly crosslinked or joined by short peptide bridges. The peptide side chains are synthesised at the inner membrane as pentapeptides and may be cleaved into shorter fragments by a number of peptidases. Peptidases may be endopeptidases or carboxypeptidases depending on whether they cleave an internal or C-terminal amino acid, respectively. Peptidases are also classified by whether they hydrolyse the bond between two D-amino acids (DD) or between a L-amino acid and a D-amino acid (LD or DL). The number and length of peptides attached to the glycan backbone provide unique muropeptide profiles for each bacterium. The PG modification pathway in bacteria is known to contain a wide array of carboxy- and endopeptidases responsible for cleaving monomeric, dimeric and trimeric peptides [9].

To date, only two carboxypeptidases involved in cleaving monomeric peptides have been identified in *C. jejuni*, Pgp1 [5] and Pgp2 [6]. Pgp2 is an LD-carboxypeptidase, which cleaves disaccharide tetrapeptides into tripeptides [6]. Pgp1 is a DL-carboxypeptidase, which cleaves disaccharide tripeptides into dipeptides [5]. Pgp1 activity is metal-dependent and requires the activity of Pgp2 to provide the tripeptide substrate [6]. When either of the *pgp1* or *pgp2* genes is mutated in the laboratory the muropeptide profile radically changes and helical cell shape cannot be maintained [5,6]. Loss of *pgp1* causes a decrease in dipeptides and tetrapeptides and an increase in tripeptides [5]. Loss of *pgp2* causes a decrease in dipeptides and tripeptides and an increase in tetrapeptides [6]. Furthermore, overexpression of *pgp1* in *C. jejuni* results in a kinked rod morphology, and muropeptide analysis of the *pgp1* overexpressing strain demonstrates a decrease in tripeptides and an increase in dipeptides [5]. Combined, these findings suggest that even subtle changes to proportions of peptides in the PG can affect *C. jejuni* cell shape.

Pgp2 orthologs are present in a wide range of bacteria that display helical, rod, vibroid (curved rod) or coccoid cell shapes [6]. In contrast, Pgp1 is most highly conserved in helical and vibroid species of the Epsilon- and Delta-proteobacteria [5]. The Pgp1 ortholog in *H. pylori*, Csd4, has also been characterised as a necessary determinant of cell shape in this helical pathogen. A defined *csd4* mutant in *H. pylori* generates a rod-shaped strain that exhibits a similar muropeptide profile to Δ*pgp1* in *C. jejuni* [5,10]. The conserved nature of Pgp1 in particular supports the hypothesis that this protein is fundamental to cell curvature and helical cell shape.

While it is known that peptidases can be redundant [11,12], single and double knockouts of Pgp1 and Pgp2 do not demonstrate any change to levels of peptide crosslinking [5,6], suggesting that there remain unidentified PG peptidases in *C. jejuni*. Thus, further identification and characterisation of the enzymes involved in PG synthesis and modification systems and how these enzymes are localised and regulated is required before we can fully understand how helical shape is generated in *C. jejuni*.

We recently performed a visual screen of 1933 transposon (Tn) mutants of *C. jejuni* for changes in cell morphology [13]. Whole genome sequence (WGS) data of the Tn mutants with altered cell shape, directed mutants, wild type (WT) stocks and isolated helical and rod-shaped 'WT' *C. jejuni*, identified a number of different genetic mutations in *pgp1* and *pgp2*, which result in a change in helical to rod bacterial cell shape [13]. In addition, we identified an isolate with a loss of curvature. In this study, we report the genome change leading to the loss of curvature and initial characterisation of the gene.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

C. jejuni strains were routinely cultured on Mueller Hinton (MH) agar (Oxoid) supplemented with 5% defibrinated horse blood (Thermo Scientific) and 5 μg/ml trimethoprim (Tp). Defined mutants and complemented strains were selected on 10 μg/ml chloramphenicol (Cm) or 50 μg/ml kanamycin (Km), as appropriate. *C. jejuni* cultures were grown in standard microaerophilic conditions (5% CO_2, 5% H_2, 85% N_2, 5% O_2) at 42 °C, unless otherwise indicated. Electrocompetent *Escherichia coli* and *C. jejuni* used in cloning were prepared and transformed as previously described [14]. Bacterial strains and plasmids used in this study are detailed in Table 1.

2.2. DNA sequencing

Sanger sequencing was performed by Source BioScience LifeSciences. WGS was performed at the Wellcome Trust Sanger Institute. Isolates were sequenced as multiplex libraries with 100 or 150 base paired-end reads using next-generation Illumina HiSeq® or MiSeq® sequencing technology, respectively. *De novo* draft assemblies were created using Velvet v1.2.08 or v1.2.10 [21] and sequencing reads were mapped to the reference genome using SMALT v.0.6.4 and v.0.7.4 [22]. SNPs and INDELs were called using SAMtools mpileup [23].

2.3. Recombinant DNA techniques

Standard methods were used for molecular cloning [24]. Chromosomal and plasmid DNA purification, DNA modification and ligations were performed using commercial kits according to the manufacturers' instructions (QIAGEN, Thermo Scientific, New England Biolabs). DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). PCR primers were purchased from Sigma (Sigma-Genosys). Thermal cycling was performed in a Gene Amp® PCR System 9700 (PE Applied Biosystems) or T100™ Thermal Cycler (Bio-Rad). Thermal cycling conditions were 96 °C for 2 min, then 30 cycles at 96 °C for 1 min, 55–60 °C for 1 min and 72 °C for 30 s/kb, and finally an extension at 72 °C for 5 min.

2.4. Generation of C. jejuni defined gene deletion mutants and complemented strains

Targeted gene deletions of *CJJ81176_1105* and *CJM1_1064* were performed by exchanging the gene with a chloramphenicol acetyltransferase (*cat*) cassette from pRY111 [19]. The *cat* cassette was amplified with primers containing *Pst*I (dare010) or *Sac*I (dare011) restriction endonuclease (RE) target sites. Flanking regions of *CJJ81176_1105* and *CJM1_10643* were amplified using upstream and downstream primers (dare_1001 to 4) containing RE sites matched to the *cat* cassette primers. PCR-amplified fragments were ligated to pUC19 prior to transformation into *E. coli*. Purified plasmid DNA was used to naturally transform *C. jejuni*. The correct genomic