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Morphology heterogeneity within a Campylobacter jejuni helical population: the use of calcofluor white to generate rod-shaped C. jejuni 81-176 clones and the genetic determinants responsible for differences in morphology within 11168 strains

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Summary

Campylobacter jejuni helical shape is important for colonization and host interactions with straight mutants having altered biological properties. Passage on calcofluor white (CFW) resulted in C. jejuni 81-176 isolates with morphology changes: either a straight morphology from frameshift mutations and single nucleotide polymorphisms in peptidoglycan hydrolase genes pgp1 or pgp2 or a reduction in curvature due a frameshift mutation in cjj81176_1105, a putative peptidoglycan endopeptidase. Shape defects were restored by complementation. Whole genome sequencing of CFW-passaged strains showed no specific changes correlating to CFW exposure. The cjj81176_1279 (recR; recombinational DNA repair) and cjj81176_1449 (unknown function) genes were highly variable in all 81-176 strains sequenced. A frameshift mutation in pgp1 of our laboratory isolate of the straight genome sequenced variant of 11168 (11168-GS) was also identified. The PG muropeptide profile of 11168-GS was identical to that of Δpgp1 in the original minimally passaged 11168 strain (11168-O). Introduction of wild type pgp1 into 11168-GS did not restore helical morphology. The recR gene was also highly variable in 11168 strains. Microbial cell-to-cell heterogeneity is proposed as a mechanism of ensuring bacterial survival in sub-optimal conditions. In certain environments, changes in C. jejuni morphology due to genetic heterogeneity may promote C. jejuni survival.

Introduction

Campylobacter jejuni is one of the most common causes of human bacterial diarrheal diseases worldwide (Epps et al., 2013). It is a Gram-negative microaerophilic organism requiring rich media for growth in vitro. Despite its metabolic limitations, it can successfully compete with the human intestinal microflora with ingestion of as few as 500 bacteria resulting in human disease (Black et al., 1988). Unlike in humans, C. jejuni colonizes the gastrointestinal tract of birds and many animal species without causing disease (Epps et al., 2013). This has a direct effect on human health with contaminated meat (primarily poultry and poultry products), unpasteurized milk and water serving as the primary sources of human infection (Epps et al., 2013).

Campylobacter jejuni has a helical morphology that, along with its polar flagella, is responsible for the characteristic corkscrew motility hypothesized to confer an advantage over rod-shaped bacteria in moving through viscous solutions, such as the mucus layer of the gastrointestinal tract (Lertsethtakarn et al., 2011).

Bacterial morphology is maintained by the peptidoglycan (PG) layer. The PG of Gram-negative bacteria is composed of strands of alternating repeat units of β1-4 linked N-acetylglicosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) cross-linked by pentapeptide side

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chains on the MurNAc residues. PG hydrolases cleaving the glycan backbone and peptide sidechains are important in the PG remodeling required to generate a particular morphology (Vollmer et al., 2008; Typas et al., 2012; Frirdich and Gaynor, 2013). As in Helicobacter pylori, C. jejuni homologs of the H. pylori CcmA, Csd1 endopeptidase, and Csd3/HdpA endo/carboxypeptidase affect the amount of curvature of the helical cell (E. Frirdich and E.C. Gaynor, unpublished). PG peptidases Pgp1 and Pgp2 are the only enzymes identified so far that result in a straight rod-shaped morphology when deleted (Frirdich et al., 2012; 2014). Pgp1 has Dl-carboxypeptidase activity cleaving PG tripeptides to dipeptides (Frirdich et al., 2012) and Pgp2 is an Lw-carboxypeptidase cleaving PG tetrapeptides to tripeptides, providing the substrate for Pgp1 (Frirdich et al., 2014). The change in shape and PG structure resulting from pgp1 and pgp2 deletions altered the biological properties of C. jejuni and how it interacts with its environment: its ability to be transmitted between hosts was compromised (displaying reduced motility and biofilm formation), it was defective in colonization in a chick model, and it showed altered activation levels of host cell receptors that differed depending on the mutant strain.

C. jejuni Δpgp1 PG hyperactivated the cytoplasmic nucleotide-binding oligomerization domain 1 (Nod1) receptor in comparison with wild type and the Δpgp1 mutant increases secretion of the IL-8 chemokine by epithelial cells, while Δpgp2 PG decreased Nod1 stimulation. The Δpgp2 mutant had no effect on IL-8 secretion levels (Frirdich et al., 2012; 2014).

There have been reports in the literature of straight C. jejuni strains arising from helical strains, although the basis of the altered morphology in these cases has yet to be established. Passage of an avirulent C. jejuni strain through chick embryos led to the isolation of more virulent rod-shaped strains better able to resist phagocytosis and survive in vivo (Field et al., 1991; 1993). In another study, various C. jejuni strains were used to colonize chickens and the strains were reexamined after passage through the chicken gut. The chicken isolates of a strongly colonizing helical bovine strain 305/94 were rod-shaped (Hanel et al., 2009). Some flagellar mutants have been described as having a straight morphology (Wassenaar et al., 1991; Matz et al., 2002; Fernando et al., 2007), although in other instances the same mutation has also resulted in no morphology changes (Hendrixson and DiRita, 2003, Joslin and Hendrixson, 2009; E. Frirdich and E.C. Gaynor, unpublished), indicating that the morphology changes were unlinked to the flagellar mutants and occurring spontaneously. Spontaneous straight variants arising after laboratory passage of C. jejuni strain 11168 and Campylobacter coli have also been reported (Gaynor et al., 2004, Ziprin et al., 2005). The complete genome sequence of a rod-shaped C. jejuni variant revealed a nucleotide deletion and subsequent truncation of the pp01 gene product (Gunther IV et al., 2015) important in helical shape determination (Frirdich et al., 2012), as described below. The presence of these numerous straight mutants would suggest that genes involved in the determination or regulation of Campylobacter helical shape may be undergoing phase variation or accumulating nonsense or missense mutations resulting in non-functional proteins. Selection of these mutants indicates that a rod-shaped morphology accompanied with the underlying PG changes may provide an advantage to C. jejuni survival under certain growth conditions.

The pp01 gene was identified in a transposon (Tn) mutant library screen on calcofluor white (CFW) for hypofluorescent or dim mutants (Frirdich et al., 2012). CFW binds β1-3 and β1-4 carbohydrate linkages such as those found in several bacterial cell surface carbohydrates and fluoresces under long wave UV light (Rattee and Breur, 1974, Wood, 1980). Despite the unknown nature of the exact C. jejuni surface carbohydrate contributing to CFW hypo- and hyperfluorescence, mutants displaying altered reactivity to CFW have been found to also display changes in stress survival and pathogenesis phenotypes (McLennan et al., 2008; Naito et al., 2010; Frirdich et al., 2012; E. Frirdich and E.C. Gaynor, unpublished). In the Tn mutant library screen on CFW that identified pp01 as having a role in maintaining helical cell shape, other Tn mutants were identified with altered cell morphology. However, unlike with pp01, the cell shape of these mutants was unlinked to the Tn insertion. This study examined the genetic basis for these shape changes. Of the 8 mutants, 6 had frameshift mutations resulting in non-functional copies of pp01, and two had mutations in cjb8176_1105 [the homolog of the H. pylori csd1 gene encoding a PG peptidase involved in determining cell curvature in that organism (Synergy et al., 2010)]. These were likely generated by passage on CFW, as passage of the wild type 81-176 strain on CFW and not on media lacking CFW, also resulted in rod-shape variants from mutations in pp01 or pgp2. The genetic variation arising from the passage of C. jejuni 81-176 on CFW was examined by whole genome sequencing (WGS), as was that of C. jejuni 11168-GS (genome sequenced) that has a rod-shaped morphology and is the laboratory passaged variant of the 11168-O (original) helical strain (Gaynor et al., 2004). The rod-shaped morphology of 11168-GS was also found to be attributed to a frameshift mutation in 11168 pp01. Our results show that due to mutations in pp01 and pgp2 and their effect on PG structure, C. jejuni can alter its shape to a straight rod. These changes in shape would also alter the biological
properties of the organism and may be advantageous to an aspect of its lifecycle either in environmental survival, transmission, colonization or infection that remains to be determined.

Results

Straight Tn mutants isolated due to their hypofluorescent phenotype on CFW resulted from mutations in ppp1 and CFW hypofluorescent Tn mutants with decreased curvature from mutations in cjj81176_1105

A mariner Tn mutant library was screened on CFW for hypofluorescent or dim mutants as part of a previous study (Frirdich et al., 2012). Of the approximately 400 dim mutants isolated, 8 mapped to distinct regions of the cjj81176_1344 gene which was renamed ppp1 (peptidoglycan peptidase 1) for its role as a PG α-carboxypeptidase (Frirdich et al., 2012). Deletion of ppp1 resulted in a straight morphology and loss of the characteristic C. jejuni helical cell shape (Frirdich et al., 2012). The role of Pgp1 in C. jejuni PG biosynthesis and its effect on C. jejuni biology were described as the basis of a previous study (Frirdich et al., 2012). In addition to the mutants in ppp1, several other Tn mutants displayed an alteration in C. jejuni cell morphology resulting in cell straightening (Fig. 1A column I, Table 1): some were straight rods (dim118, dim120, dim122, dim129, dim132, and dim133) and others were still helical, but displayed a decreased amount of curvature (dim111 and dim128). The location of the Tn insertions were mapped as described previously (Frirdich et al., 2012), with no obvious candidates in genes predicted to affect cell morphology (Table 1).

Identifying the genetic basis for hypofluorescence in dim111, dim118, dim120, dim122, dim128, dim129, dim132, and dim133

To determine whether the changes in cell morphology in dim mutants dim111, dim118, dim120, dim122, dim128, dim129, dim132, and dim133 were linked to the Tn insertion, the wild type C. jejuni helical 81-176 strain was transformed with genomic DNA from the dim mutants and plated on the antibiotic corresponding to the antibiotic resistance cassette encoded by the mariner Tn antibiotic resistance cassette to remake the mutations. Unlike with the Tn insertions in ppp1, the straightened morphology (Fig. 1A column II; Table 1) and the dim phenotype dim111, dim118, dim120, dim122, dim128, dim129, dim132, and dim133 mutants was not retained in the remake strains. Since ppp1 and ppp2 are the only C. jejuni genes known to produce rod-shaped cells when deleted, the ppp1 and ppp2 genes in each dim mutant were sequenced to determine whether changes in either of these genes was affecting the morphology of the mutants. Nucleotide insertions or deletions were detected in ppp1 in dim118, 120, 122, 129, 132, and 133 (Table 1, Supporting Information Fig. S1). Four different mutations were identified: a deletion from A8 to A7 in a poly-A tract starting at nucleotide 1184 (dim120), an increase from G4 to G5 in a poly-G tract at nucleotide 383 (dim118 and dim129), a deletion of a G from a GATGC repeat sequence at nucleotide 412 (dim122 and dim133), and an insertion from A3 to A4 in a poly-A tract starting at nucleotide 1077 (dim132). These changes resulted in frameshift mutations introducing premature stop codons and truncated versions of Pgp1 (Table 1). The nucleotide deletion in dim118 and dim129 was identical to that identified in the ppp1 gene of C. jejuni RM1285 (Gunther IV et al., 2015). The ppp2 gene sequence was identical to wild type in all mutants.

The wild type 81-176 ppp1 gene was introduced into the dim mutants to ascertain whether the changes in morphology could be restored. Complementation analysis with ppp1 is complicated by the fact that (1) ppp1 copy number affects complementation (increased levels of ppp1 results in changes in morphology); and (2) the ppp1 gene is located in the middle of a putative operon so the location of the ppp1 promoter is unclear (Frirdich et al., 2012). Transcriptional start sites predicted by C. jejuni transcriptome analysis (Dugar et al., 2013) are indicated in Fig. 1B. Plasmids pEF35R and pEF38R were used for complementation analysis and contain ppp1 with varying amounts of the upstream region (Fig. 1B) cloned into the pRRC (CmR) plasmid (Karlyshev and Wren, 2005). The ppp1 region was cloned in the reverse orientation to the catR cassette to avoid overexpression from the cat promoter and integrated into the rRNA spacer region. The pEF38R construct expressing ppp1 and cjj81176_1345 is the minimal construct that complements the shape defect in a Δppp1 mutant (data not shown); and the plasmid pEF35R contains the entire upstream region of ppp1 (cjj81176_1345-cjj81176_1348) and complements all the Δppp1 mutant phenotypes to wild type (Frirdich et al., 2012). Wild type morphology was partially restored in dim118, dim120, dim122, dim129, dim132, and dim133 with the introduction of ppp1-1345 (from pEF38R) and completely restored with ppp1-1348 (from pEF35R) (Fig. 1A, Table 1). Complementation of the dim118 and dim129 morphology defect with wild type ppp1 indicates that the rod shaped morphology of the C. jejuni strain described by Gunther IV et al. (2015) (with a similar mutation in ppp1 as dim118 and dim129) could likely also be complemented by wild type ppp1, although complementation analysis is required to confirm this. The morphology of
**Fig. 1.** *Campylobacter jejuni* 81-176 Tn mutants isolated from a screen on CFW for their *dim* phenotype exhibited a change in morphology unlinked to the Tn insertion.

A. DIC microscope images of *C. jejuni* wild type 81-176 and *dim* mutants grown on solid media for 18 h. Column I shows DIC images of the wild type 81-176 strain and the *dim* mutants isolated from a screen on CFW which exhibited a loss in cell curvature and straightened cell morphology that were not previously reported. The rod-shaped *dim100* mutant having an insertion in the *cjj81176_1344* gene (renamed *pgp1*) was previously published (Frirdich *et al.*, 2012). The DIC images depicted in column II represent the morphology of the wild type strain 81-176 strain transformed with genomic DNA from the respective *dim* mutants and selected for by the Km antibiotic resistance marker carried by the Tn to remake the mutations. These strains did not retain the changes in morphology seen after the mutants were isolated on CFW, indicating that the original changes in morphology were unlinked to the Tn insertion, which was not the case with *dim100* (Frirdich *et al.*, 2012). The DIC images in columns III and IV are those of the original *dim* mutants expressing *pgp1* and varying amounts of the upstream region of the *pgp1* gene cluster in the rRNA spacer region of the chromosome. The plasmids used for this complementation analysis are shown in panel B.

B. *C. jejuni* 81-176 *pgp1* gene locus. The regions cloned into the integrative vector pRRC (CmR) to form plasmids pEF35R and pEF38R used for complementation analysis (Panel A, columns III and IV) are shown below the gene cluster. An R after the plasmid name indicates that the region is cloned in the opposite direction as the antibiotic resistance cassette promoter. Transcriptional start sites as predicted by *C. jejuni* transcriptome analysis carried out by Dugar *et al.* (2013) are designated by an asterisk and an arrow indicating the direction of transcription.

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Table 1. Summary of the nucleotide changes in the pgp1 gene of the dim mutants isolated from a Tn mutant library screen on CFW and cellular morphology of the mutants, retransformed mutants, and original mutants expressing pgp1 and varying amounts of the upstream region of pgp1 (as the promoter of pgp1 is unknown) displayed in Fig. 1.

<table>
<thead>
<tr>
<th>Location of Tn insertion</th>
<th>Description of mutation in pgp1 (1395 bp)</th>
<th>Sequence surrounding mutation</th>
<th>Predicted length of Pgp1 (amino acids)</th>
<th>Shape of retransformed mutant</th>
<th>Shape of mutant expressing pgp1-1345 at the rRNA locus</th>
<th>Shape of mutant expressing pgp1-1348 at the rRNA locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>dim111, dim128</td>
<td>Cjj81176_0478/ThiC Thiamine biosynthesis protein</td>
<td>Wild type pgp1</td>
<td>Not applicable</td>
<td>464</td>
<td>Very slightly helical</td>
<td>Very slightly helical</td>
</tr>
<tr>
<td>dim118</td>
<td>Cjj8176_pTet0039/cmbg_11 VirB11-like ATPase</td>
<td>A deleted at NT 1187</td>
<td>AAA-AAAA (A tract)</td>
<td>402</td>
<td>Straight</td>
<td>Helical</td>
</tr>
<tr>
<td>dim120</td>
<td>Cjj8176_pVir0003 VirB10 homolog (similar to VirB)</td>
<td>G inserted after NT 386</td>
<td>GGGGG (G tract)</td>
<td>131</td>
<td>Straight</td>
<td>Helical</td>
</tr>
<tr>
<td>dim122</td>
<td>Cjj8176_0307 Transaldolase</td>
<td>G deleted at NT 412</td>
<td>GAT-CGATGC (GATGC repeat)</td>
<td>149</td>
<td>Straight</td>
<td>Slightly curved</td>
</tr>
<tr>
<td>dim129</td>
<td>Cjj81176_0315 PEB3</td>
<td>A deleted at NT 1187</td>
<td>AAA-AAAA (A tract)</td>
<td>402</td>
<td>Straight</td>
<td>Slightly curved, few straight</td>
</tr>
<tr>
<td>dim132</td>
<td>Cjj81176_0206/7 Hypothetical protein</td>
<td>A inserted after NT 1077</td>
<td>AAAA (A tract)</td>
<td>372</td>
<td>Straight</td>
<td>Helical, few straight</td>
</tr>
<tr>
<td>dim133</td>
<td>Cjj81176_0307 Transaldolase</td>
<td>G deleted at NT 412</td>
<td>GAT-CGATGC (GATGC repeat)</td>
<td>149</td>
<td>Straight</td>
<td>Slightly curved</td>
</tr>
</tbody>
</table>

a. The results of pgp1 sequencing. The sequence of pgp2 was identical to that of wild type.
b. The full-length Pgp1 is 464 amino acids. Frameshift mutations introduce premature STOP codons.
c. Summary of column I Fig. 1A.
d. The wild type C. jejuni 81-176 strain was transformed with genomic DNA from the respective dim mutants and selected for by the Km antibiotic resistance marker carried by the Tn to remake the mutations in a clean background. Summary of column II Fig. 1A.
e. The 1344-1345 region from pEF35R (Fig. 1B) was integrated into the rRNA spacer region. Summary of column III Fig. 1A.
f. The 1344-1348 region from pEF35R (Fig. 1B) was integrated into the rRNA spacer region. Summary of column IV Fig. 1A.
g. The dim111 and dim128 mutants have a deletion in cjj81176_1105 deleting NT 743-746 (ACCT) resulting in a frameshift mutation and premature stop codon (Supporting Information Table S3).
dim111 and dim128 was unchanged with the introduction of pgp1, as expected, since these mutants did not have a mutated copy of pgp1 (Fig. 1A, Table 1). WGS of dim111 and dim128 identified a deletion in cjj81176_1105 deleting nucleotides 743-746 (-ACCT) resulting in a frameshift mutation and premature stop codon (Supporting Information Table S3). The cjj81176_1105 gene product is a homolog of H. pylori Csd1 (30% identity/52% similarity). Csd1 is a PG endopeptidase involved in determination of cell curvature in H. pylori (Sycuro et al., 2010). The morphology of a mutant in cjj81176_1105 resembled that of dim111 and dim128 with cell straightening, but not a complete loss of curvature (Supporting Information Fig. S2). Introduction of 1105 from pRRC-1105 into the rRNA locus of dim111 and dim128 restored the morphology defect of those mutants (Supporting Information Fig. S2).

Selection of straight C. jejuni 81-176 isolates from a helical wild type population by passage on CFW

Since a Tn mutant library screen on CFW selected for mutants with changes in cell morphology unlinked to the Tn insertion, the C. jejuni wild type strain 81-176 was passaged on CFW to determine whether it was the passage on CFW that generates or selects for straight isolates. A similar protocol to the original CFW screen (Fridrich et al., 2012) was used and is outlined in Fig. 2 with the exception that colonies were randomly selected and not selected by fluorescence. Two screens on CFW were carried out (Fig. 2A and B). In the first screen (Fig. 2A), a total of 100 colonies were carried through the screen. The controls plated on BHI only for 1 or 2 passages were all helical. The colonies exposed to CFW were examined for shape only after a second passage either on BHI-CFW or BHI alone and only after 1 day of growth (Fig. 2A). Of the 100 colonies, 14 displayed a mixture of cell morphologies with both helical and straight cells after one passage on CFW, regardless of whether the second passage was on BHI-CFW or BHI. One of these colonies (colony 12) was re-streaked for isolated colonies [colony #12 for screen I (A), and colonies #25 and 33 for screen II (B)] and the morphology of these were re-examined. Of these, colonies selected for further analysis are described in the figure.
For the second screen, fewer colonies (50 rather than 100), but more conditions were tested for their effect on morphology (Fig. 2B). As in the first screen, all colonies plated on BHI alone remained helical (Fig. 2B). After the first passage on BHI-CFW, all 50 colonies were helical (Fig. 2B). After a second passage on either BHI or BHI-CFW, colony 25 had helical and straight cells in the population after 1 day of growth (Fig. 2B), with more straight cells after growth on CFW. After 2 days of growth on BHI and CFW, straight cells appeared in the population of colony 33 (Fig. 2B). Colonies 25 and 33 were re-streaked for isolated colonies on MH. For colony 25, of the 10 isolated colonies examined, 2 were helical and 8 were straight (Fig. 2B). Straight colony 25-1 and helical colony 25-6 were selected for further analysis (Fig. 3A). Increasing the amount of CFW added to the BHI plates up to tenfold had no effect on viability and did not increase the observed number of colonies displaying a straight morphology.

**Straight C. jejuni 81-176 isolates from passage on CFW result from point mutations in pgp1 and pgp2**

To determine whether the straight morphology of CFW-passaged *C. jejuni* 81-176 isolates were due to changes in *pgp1* or *pgp2*, these genes were sequenced in 12-1, 12-9, 12-10, 25-1, 25-6, 33-1 and 33-2. Isolates 12-1, 12-9, and 33-1 all had single nucleotide polymorphisms (SNPs) in *pgp1* and *pgp2* genes were sequenced in all isolates. Nucleotide differences resulting in amino acid changes are indicated in brackets. For complementation analysis, the *pgp1* or *pgp2* genes were introduced into the rRNA spacer region in isolates in which a mutation was identified. The *C. jejuni* 81-176 region used for *pgp1* and *pgp2* complementation was that from plasmid pEF38R indicated in Fig. 1B and pJV4 described in (Fridich et al., 2014) respectively. B. complementation analysis of *pgp1* and *pgp2* mutations demonstrating the lack of function of the single amino acid changes in isolates 12-1, 25-1, and 33-1. The *C. jejuni* 81-176 *pgp1* region inserted into the rRNA spacer region was that from pEF38R (Fig. 1B) and the 12-1 and 33-1 *pgp1* regions were identical to the region used in pEF38R forming plasmids pEF82R and pEF80R respectively. The *C. jejuni* 81-176 *pgp2* gene and upstream region was that of pJV4 and a similar region from 25-1 that was cloned into pEF81.

**Fig. 3.** Cellular morphology of *C. jejuni* 81-176 straight isolates from CFW passaging and complementation analysis. DIC microscope images of *C. jejuni* strains grown on solid media for 18 h. A. cellular morphology of isolates with a straight and helical phenotype from colonies #12, 25, and 33 isolated from passaging of *C. jejuni* 81-176 on CFW (Fig. 2). The *pgp1* and *pgp2* genes were sequenced in all isolates.
These mutations result in single amino acid changes (Supporting Information Figs S1 and S3). The *pgp1* and *pgp2* sequences in helical isolates were identical to those of the wild type 81-176 (Supporting Information Table S3). Since 12-1 and 12-9 had identical mutations, only 12-1 was used for further analysis.

The wild type 81-176 *pgp1* and *pgp2* genes were introduced into the rRNA spacer region of the corresponding SNP-containing isolates to determine if the mutations resulted in the shape phenotype. The *C. jejuni* 81-176 region used for *pgp1* and *pgp2* complementation was that from plasmid pEF38R indicated in Fig. 1B and pJV4 described previously (Fridrich et al., 2014) respectively. Expression of wild type *pgp1* in isolates 12-1 and 33-1 and wild type *pgp2* in isolate 25-1 restored wild type helical morphology (Fig. 3A). The mutated 12-1 and 33-1 *pgp1* alleles and upstream sequence equivalent to the *pgp1* region in pEF38R (Fig. 1A) were cloned into pRRC in the reverse orientation, as in pEF38R. Introduction of the mutated 12-1 and 33-1 *pgp1* alleles could not restore the helical shape in a *pgp1* deletion strain (Fig. 3B). The 25-1 *pgp2* allele was cloned into pRRC using the primers used to generate pJV4 (Fridrich et al., 2014) used for *pgp2* complementation and integrated in the *pgp2* deletion strain. The 25-1 allele did not complement a *pgp2* mutant (Fig. 3B).

The muropeptide profile of the 81-176 33-1 (*Pgp1 S402F*) and 25-1 (*Pgp2 G131C*) isolates resemble that of a *pgp1* and *pgp2* deletion strain respectively.

Since 33-1 and 25-1 lack a functional *pgp1* and *pgp2* allele, respectively, it was expected that the muropeptide profile would be identical to that of the corresponding deletion mutant. PG was isolated from *C. jejuni* 81-176 wild type, 25-1, 25-6, 33-1, and 33-2, and the muropeptides were separated by HPLC (Table 2, Supporting Information Table S4). The muropeptide changes between the straight isolates and wild type were compared with those of ∆*pgp1* and ∆*pgp2* analyzed previously (Fridrich et al., 2012; 2014). The 25-1 isolate had a similar muropeptide profile to that of ∆*pgp2* with decreased dipeptides, no tripeptides, and increased tetrapeptides. The 33-1 isolate muropeptide profile resembled that of ∆*pgp1* with similar changes in muropeptide species: a decrease in dipeptides, an increase in tripeptides and decrease in tetrapeptides in the monomeric, dimeric, and trimeric forms. These results are consistent with the sequencing data identifying a SNP in *pgp2* in 25-1 and a SNP in 33-1 in *pgp1*. The muropeptide profile of 81-176 12-1 was not analyzed, but would likely resemble that of 33-1. Helical 81-176 that had been passaged on CFW (isolates 25-6 and 33-2) showed a relative increase in O-acetylation levels and decrease in tripeptides in comparison with 81-176 that had been grown only on MH.

**Whole genome sequencing (WGS) to examine the effect of growth on CFW**

WGS was used to determine whether CFW was having mutagenic effects on our *C. jejuni* strains. Genomic comparisons were carried out between 81-176 strains passaged on CFW (*dim111, dim128, 12-1, 12-10, 25-1, 25-6, 33-1, and 33-2*) to our non-CFW passaged wild type. In addition, WGS was used to compare our laboratory strain of *C. jejuni* 81-176 to the published sequence available in GenBank (NC_008787).

There were a total of 32 variants in 14 different genes and 9 in intergenic regions in our wild type 81-76 strain in comparison with the published 81-176 sequence, with 12 of the 32 changes occurring at homopolymeric tracts (Supporting Information Table S3). The *cjj81176_1449* gene of unknown function showed a large amount of variation, with 6 individual SNPs and 2 substitutions and 1 frameshift at a variant frequency ranging from 18.9% to 37.50%. The same 9 changes in *cjj81176_1449* were also present in the CFW passaged strains at a slightly higher variant frequency ranging from 26.8% to 54.7%. A SNP occurring at a variant frequency of 34.6% in the *recR* gene (*cjj81176_1279*) involved in the recombinational process of DNA repair was also present in all other sequenced strains at frequencies ranging from 27.1% to 41.1%. The *cjj81176_1449* and *recR* genes are therefore likely to be highly variable genes in the *C. jejuni* 81-176 population. Three SNPs were present at variant frequencies under 20%. SNPs in *cjj81176_0227* (*purF*), *cjj81176_1105*, and *cjj81176_1354* occurred at frequencies of 94.9%, 100% and 100%, respectively and were present in all other strains sequenced, indicating that they were present in the starting strain and maintained during passage.

The *dim* mutants sequenced (*dim111* and *dim128*) harbored a deletion in *cjj81176_1105* at a variant frequency of 95.1–99.3% (Supporting Information Table S3) and this deletion was responsible for the changes in cell shape observed in these strains (as described above). These mutants showed an accumulation of a greater total number of genomic changes (57, *dim111*; and 53, *dim128*) than the 81-176 strains passaged on CFW (34 in 12-1; 32 in 12-10; 40 in 25-1; 31 in 25-6; 36 in 33-1; and 39 in 33-2) and the wild type 81-176 (32 total changes). The *dim* mutants had 10 genomic changes not present in the other strains at frequencies of 15.6–58.6% in *cjj81176_0477*, the gene encoding the DNA polymerase III subunit epsilon responsible for the
Table 2. Summary of the muropeptide composition of *C. jejuni* wild-type 81-176 passaged on CFW (25-1, 25-6, 33-1, 33-2) in comparison with 81-176 wild type, Δ*pgp1* (Frirdich et al., 2012) and Δ*pgp2* (Frirdich et al., 2014) mutant strains characterized previously.\(^a\)

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<th>(81-176)</th>
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<td>81-176</td>
<td>81-176</td>
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<td>8.0(^*)</td>
<td>15.2</td>
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\(^a\) The morphology of each strain is indicated below the strain description, as is the date of analysis and the strain to which comparisons were made.
\(^b\) Numbers represent the percent area of each muropeptide from Table S4 calculated to give a total of 100%. Values indicated with an asterisk (*) or both bolded and with an asterisk, represent a greater than or equal to 20% or 30% difference, respectively in comparison with the strain mentioned in the table.
\(^c\) The values for the percentage of O-acetylated species do not represent the true level of O-acetylation in these strains, as most of these substitutions are lost in the standard alkaline reduction procedure used in this study to prepare the PG. These values were included to demonstrate the relative difference in O-acetylation between the samples.
\(^d\) nd = not determined.
3'–5' exonuclease activity. The dim mutants also showed the occurrence of more SNPs in cij81176_1397, the feoB gene responsible for ferrous iron acquisition (Nakake et al., 2006) with strains 25-6 and 33-2 having one SNP in feoB. All these SNPs occurred at very low frequencies (<25%). Excluding the SNPs in pgp1 (in 12-1 and 33-1) and pgp2 (in 25-1) that occurred at 98.9–100%, the 81-176 strains passaged on CFW (12-1, 12-10, 25-1, 25-6, 33-1, and 33-2) did have a few genomic changes not present in the wild type strain, but they were not consistent across the strains and occurred at low frequencies (11 of them <25% and 3 of them <36%) (Supporting Information Table S3).

The pgp1 gene of the straight 11168-GS (genome sequenced) strain is non-functional

The C. jejuni NCTC11168 strain was originally isolated from a human diarrheic patient in 1977 [11168-O (original)] (Skirrow, 1977) and then sequenced in 2000 [11168-GS (genome sequenced)] (Parkhill et al., 2000). The 11168-O and 11168-GS strains have several phenotypic differences decreasing the virulence of the 11168-GS strain (Gaynor et al., 2004). The 11168-O strain is helical while the laboratory passaged 11168-GS variant has lost its helical morphology and is rod-shaped, similar to a pgp1 mutant constructed in 11168-O (Fig. 4A). The pgp1 and pgp2 genes of 11168-GS and 11168-O were sequenced and compared with the NCTC 11168 sequence deposited in GenBank (accession number NC_002163). A G nucleotide was deleted in pgp1 at position 157 of a total of 1395 nucleotides in the 11168-GS from our laboratory collection (Supporting Information Fig. S1), but not the 11168 sequence deposited in GenBank (Supporting Information Table S5). This change resulted in a frameshift mutation and truncated Pgp1 protein (the predicted length of Pgp1 is 56 amino acids in comparison with the 464 amino acid full-length Pgp1). The pgp2 gene sequences were identical in all strains (Supporting Information Table S5). To demonstrate that 11168-GS pgp1 was non-functional, the 11168-GS pgp1 gene was expressed in 81-176 Δpgp1. Unlike the 11168-O pgp1 gene, 11168-GS pgp1 could not complement a 81-176 Δpgp1 mutant (Fig. 4B). Despite 7 amino acid differences between the pgp1 gene of 81-176 and 11168-O (Supporting Information Fig. S1), either gene could complement the shape phenotype of 11168-O-Δpgp1 (Fig. 4A). Neither 81-176 nor 11168-O pgp1 alone could restore the wild type helical shape of 11168-GS; however, some of the cells did regain a very slight degree of helicity (Fig. 4A). WGS was carried out to determine whether another peptidase was affected in 11168-GS that would affect complementation to wild type morphology (see below).

A 11168-O strain microaerophilically passaged 13 times reported previously by our laboratory (Gaynor et al., 2004), referred to here as 11168-M (microaerophilically passaged), has a straight morphology due to a deletion in pgp1 resulting in a premature stop codon. For complementation analysis, the pgp1 gene from 11168-O and 81-76 was introduced at the rRNA spacer region. The C. jejuni 81-176 region used for pgp1 complementation was that from plasmid pEF38R indicated in Fig. 1B. A similar region from 11168 was used.


was affected in 11168-GS that would affect complementation to wild type morphology (see below).
Fig. 5. HPLC elution profile of C. jejuni 11168-O, 11168-GS, and 11168-\(\Delta\)pgp1 muropeptides. Purified PG from strains all grown for 1 day was digested with cellosyl and the resulting muropeptides were reduced with sodium borohydride and separated on a Prontosil 120-3-C18 AQ reverse-phase column. HPLC profiles are shown for A, C. jejuni 11168-O strain with helical morphology; B, 11168-GS strain with straight morphology; and C, the 11168-\(\Delta\)pgp1 mutant strain with straight morphology. Peak numbers correspond to the main muropeptide peak fractions of C. jejuni 81-176 analyzed by LTQ-FT-MS (Fridrich et al., 2012), with two additionally characterized peaks, and shown in panel D. G, N-acetylgalactosamine; M, reduced N-acetylmuramic acid; L-Ala, L-alanine; D-iGlu, D-isoglutamic acid; D-Glu, D-glutamic acid; meso-DAP, meso-diaminopimelic acid; Gly, Glycine; Ac, O-acetyl groups at the C-6 hydroxyl group of MurNAc; Anh, 1,6-anhydro group at MurNAc. The asterisk (*) indicates that it is not known on which MurNAc residue the modification occurs.
The morphology defect could be complemented back to a wild type helical morphology by introduction of \( \text{pgp1} \) (Fig. 4A). It should be noted that the exact nature of how this strain was generated is unknown, as repeated attempts at passaging 11168-O over 25 times under microaerophilic conditions did not result in a straight strain.

### Table 3. Summary of the muropeptide composition of the \( C. \text{jejuni} \) wild-type 11168-O (original), 11168-GS (genome sequenced) and 11168-O-\( \Delta \text{pgp1} \) mutant strain. The 81-176-\( \Delta \text{pgp1} \) (Frirdich et al., 2012) mutant strain used for comparison was characterized previously.a

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<th>11168-GS</th>
<th>11168-GS</th>
<th>11168-O</th>
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<td>Pentapeptides (Total)</td>
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<td>0.9*</td>
<td>0.9*</td>
<td>1.4</td>
<td>1.1*</td>
<td>1.2</td>
<td>0.7</td>
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<tr>
<td>Acetylated (Total)c</td>
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<td>10.4*</td>
<td>10.4*</td>
<td>3.1</td>
<td>1.1*</td>
<td>3.5</td>
<td>5.7</td>
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<td>Anhydro chain ends (Total)</td>
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<td>7.0</td>
<td>7.0</td>
<td>8.0</td>
<td>7.9</td>
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<td>Average chain length</td>
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<td>12.4</td>
<td>12.7</td>
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<td>Degree of cross-linkage</td>
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<td>27.3</td>
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<tr>
<td>% Peptides in cross-links</td>
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<td>57.4</td>
<td>61.3</td>
<td>57.8</td>
<td>56.7</td>
</tr>
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</table>

a. The morphology of each strain is indicated below the strain description, as is the date of analysis and the strain to which comparisons were made.

b. Numbers represent the percent area of each muropeptide from Table S4 calculated to give a total of 100%. Values indicated with an asterisk (*) or both bolded and with an asterisk, represent a greater than or equal to 20% or 30% difference, respectively in comparison with the strain mentioned in the table.

c. The values for the percentage of O-acetylated species do not represent the true level of O-acetylation in these strains, as most of these substitutions are lost in the standard alkaline reduction procedure used in this study to prepare the PG. These values were included to demonstrate the relative difference in O-acetylation between the samples.

d. nd = not determined.
The 11168-GS muropeptide profile is similar to that of 11168-O-Δpgp1
PG was isolated from 11168-GS, 11168-O, and 11168-O-Δpgp1 and the muropeptides were separated by HPLC (Fig. 5, Table 3, Supporting Information Table S4) to compare the changes between helical 11168-O and straight 11168-GS and whether these were consistent with the changes in a 11168-O-Δpgp1 mutant. As with 81-176 Δpgp1, 11168-GS and 11168-O-Δpgp1 showed a decrease in dipeptides, increase in tripeptides and decrease in tetrapeptides in both the monomeric and multimeric forms. Despite the inability of pgp1 to completely restore the helical shape of 11168-GS, 11168-O-Δpgp1 had very similar muropeptide profiles. The relative levels of O-acetylation in 11168-GS were 5.1 fold higher than 11168-O, while 11168-O-Δpgp1 O-acetylation was 2.8-fold lower than 11168-O. Analysis of the O-acetylation levels would have to be carried out to determine the exact differences, as these substitutions are lost by the standard alkaline reduction procedures used in this study to analyze the muropeptide composition. The muropeptide profiles of 11168-O and 81-176 were relatively similar.

WGS to compare 11168-O and 11168-GS and our laboratory strain of 11168-GS to the published sequence of 11168-GS
WGS allowed comparison between our laboratory strain of C. jejuni 11168-GS and the published sequence available in GenBank [NC_002163 (Parkhill et al., 2000)]. There were a total of 25 variants in 20 different genes and 2 in intergenic regions in our 11168-GS strain (Supporting Information Table S5). Of the 25 variants, 10 occurred at homopolymeric tracts, 13 were SNPs, one was the deletion in cj1345c (pgp1) and one was an insertion in cj0628 (a putative lipoprotein). Excluding the expected variants in homopolymeric tracts, 8 of the changes occurred at very low frequencies (<26%, one change in cj0628 (a putative lipoprotein) occurred at a frequency of 74.1%, and the remaining 6 [in cj0431 (putative periplasmic ATP/GTP-binding protein), cj0437 (sdhA), cj0807 (putative oxidoreductase), cj1262 (racS), cj1345c (pgp1) and cj1401c (tpiA)] occurred at frequencies >98%. The mutation in pgp1, as described above, is responsible for the straight morphology of our laboratory strain.

Our laboratory 11168-O strain had 36 changes in 28 different genes in comparison with the 11168-GS published sequence, with 5 of the changes in intergenic regions. Of the 36 changes, 20 were in homopolymeric tracts, 15 were SNPs, and there was one insertion in cj0945c (a putative helicase). Excluding the changes in homopolymeric tracts or in intergenic regions, 8 of the changes occurred at very low frequencies of <34%, one change in cj1259 (porA, the major outer membrane protein) occurred at a frequency of 71.3%, and the remaining 5 [in cj0276 (mreB), cj0284c (cheA), cj0431 (putative periplasmic ATP/GTP-binding protein), cj0455 (novel glycopeptide), and cj0807 (putative oxidoreductase)] occurred at frequencies >98% (summarized in Supporting Information Table S6). There were 6 changes common to both our laboratory 11168-O and 11168-GS strains: changes in cj0431, cj0807, cj0935c (2 changes; putative sodium:amino-acid symporter family protein), cj1263 (recR), and cj1437c (aminotransferase). Changes in cj0276 (mreB), cj0284c (cheA), cj0431 (putative periplasmic ATP/GTP-binding protein), cj0455 (novel glycopeptide), cj0807 (putative oxidoreductase), and cj1259 (porA, the major outer membrane) also occurred in NCTC 11168-BN148, a laboratory variant of 11168-GS obtained from the Centers of Disease Control in 1979 and with invasion, motility and morphology phenotypes resembling 11168-O and not 11168-GS (Revez et al., 2012). As seen in the genome sequencing of 81-176, the recR gene was also variable in 11168. The C. jejuni 11168 homolog (cj1456c) of the 81-176 gene (cjj81176_1449) that was variable in 81-176 was not variable in 11168.

The motile strains 11168-O and 11168-BN148 (Revez et al., 2012; Supporting Information Table S5) had a SNP in cj0284c (cheA) resulting in an amino acid substitution of I290T that was not present in our laboratory 11168-GS strain (non-motile) or the published 11168-GS strain. Mutants in cheA are non-motile (Golden and Acheson, 2002), so the amino acid substitution may explain the motility defect of 11168-GS.

Examination of the basis why expression of the 11168-O pgp1 gene does not restore wild-type helical morphology to 11168-GS
The 11168-GS strain expressing wild type Pgp1 produced cells with a very minimal helical pitch (Fig. 4A). This could result from: (1) differences in pgp1 expression levels affecting the levels of complementation, (2) mutation in a 11168-GS gene responsible for dictating helical pitch, or (3) alterations in the abundance of proteins affecting cell wall and cell shape biogenesis, or (3) differences in pgp1 expression levels affecting the levels of complementation. Experiments were carried out to address these hypotheses.

Differences in levels of Pgp1. Since overexpression of pgp1 as well as deletion of pgp1 results in cell straightening (Fridrich et al., 2012), the proper levels of Pgp1 are required for wild-type helical morphology. Genetic expression levels of pgp1 were examined by real-time quantitative PCR (RT-qPCR) in 11168-O, 11168-GS, 11168-GS...
Expression of the pgp1 gene is higher in a 11168-GS background than in 11168-M or 11168-O strains. RNA was extracted from rod shaped C. jejuni strains 11168-GS, 11168-GS expressing 11168-O pgp1, 11168-M, and 11168-O Δpgp1, and helical strains 11168-O, 11168-M overexpressing 11168-O pgp1, and 11168-O Δpgp1 overexpressing 11168-O pgp1 grown in broth for 24 h post-inoculation at OD 0.002. The transcript levels of the pgp1 gene were examined by q-RT-PCR and relative quantification was carried out by normalizing against unit mass (10 ng cDNA). Relative expression was calculated by the ΔCt method and fold differences are displayed in comparison with the helical 11168-O strain. Reactions were performed in triplicate on three independent samples.

Expression of 11168-O pgp1, 11168-M, and 11168-O Δpgp1 and 11168-O Δpgp1 expressing 11168-GS pgp1 (Fig. 6). Primers used for RTPCR bound outside of the area deleted in 11168-O Δpgp1. The 11168-GS strains showed much higher expression levels of pgp1 than the 11168-M and 11168-O Δpgp1 strains. Expression levels of pgp1 were identical to those of 11168-O in 11168-M, 11168-M overexpressing 11168-O pgp1, 11168-O Δpgp1, and 11168-O Δpgp1 expressing 11168-O pgp1.

Identification of genomic changes in 11168-GS potentially affecting the helical pitch of 11168-GS helical morphology. WGS of 11168-GS, 11168-O, 11168-M and the published sequence of NCTC 11168-BN148 were compared with determine whether another PG peptidase or shape determining gene could be altered in 11168-GS that would prevent complementation to wild type morphology (Supporting Information Table S5). The morphology of the sequenced strain of 11168 deposited in GenBank (Parkhill et al., 2000) is most likely helical (since it did not have a frameshift mutation in pgp1 like our laboratory version of this strain), although the degree of helical pitch in this strain is unknown. Therefore, genomic changes from Supporting Information Table S5 present in >50% of the population that are either unique to our laboratory strain of 11168-GS or unique to the helical 11168-O strain and -M strain that could be complemented to a helical morphology were identified (summarized in Supporting Information Table S6) as possible causes for the lack of complete restoration of morphology by introduction of pgp1 into 11168-GS.

Interestingly, 11168-O, 11168-M, and 11168-BN148 all had a SNP in cj0455c (mreB) (present in 98.8% and 100% of the 11168-O and 11168-M population, respectively) resulting in amino acid substitution D48G that was present in neither our laboratory 11168-GS strain nor the published 11168-GS (Supporting Information Table S6). MreB from C. jejuni 81-176 has also a Gly residue at position 48. Since MreB is generally the major determinant of cell shape in non-spherical bacteria and is involved directly or indirectly in positioning the PG synthesis machinery (reviewed in Defossez et al., 2012; Errington, 2015), it is conceivable that an amino acid change in MreB could alter its function or its ability to interact properly with its protein interaction partners thereby affecting the helical shape of C. jejuni. However, introduction of 11168-O mreB at the rRNA spacer region of 11168-GS expressing 11168-O pgp1 did not result in wild type helical morphology (data not shown). Conversely, expressing 11168-GS mreB at the rRNA spacer region in 11168-O followed by deletion of the 11168-O mreB had no effect on morphology (data not shown).

WGS also identified a SNP in the cj0455c gene of 11168-O and 11168-M present at 98.7% and 100%, respectively, that resulted in extension of the gene product by 61 amino acids (“115Q, where the asterisk represents the stop codon). In a study using proteomics and glycometabolism to compare 11168-O to 11168-GS, the cj0455c gene was found to encode a glycopeptide with >100-fold increase in 11168-O in comparison with 11168-GS (Scott et al., 2014). Cj0455c is a putative membrane protein of unknown function. Expression of 11168-O 0455 at the rRNA spacer region of 11168-GS expressing 11168-O pgp1 also had no effect on morphology (data not shown).

Our laboratory strain of 11168-GS differed from the sequenced 11168-GS strain, 11168-O and 11168-M strains in the cj1306c gene. Our laboratory variant had a frameshift mutation resulting from a deletion of a C nucleotide at a tandem repeat, resulting in a C9 to a C8 mutation leading to a premature stop codon truncating the protein from 409 amino acids to 202 amino acids. Cj1306c and the downstream gene product Cj1305c show 78%/86% identity/similarity, respectively, and both are putative hydrolases with a peptidase S28 domain. Expression of 11168-O 1305-1306 at the rRNA spacer region of 11168-GS expressing 11168-O pgp1 had no effect on morphology (data not shown).
Identification of proteomic changes potentially affecting cell wall or cell shape in 11168-GS. A proteomic study carried out by Scott et al. (2014) indicated that the Cj0796c putative PG hydrolase along with MurF (Cj0795c) were found in higher amounts in 11168-O than in 11168-GS (Scott et al., 2014). Cj0796c contains an alpha/beta hydrolase fold, as with Cj1305c. MurF (Cj0795c) is a UDP-N-acetylmuramoyl-tripeptide d-alanyl-d-alanine ligase responsible for ligating the d-Ala-d-Ala dipeptide to UDP-N-acetylmuramoyl-tripeptide generating UDP-MurNAc pentapeptide in PG precursor synthesis. Ddl (11168 Cj0798c) just upstream of 11168 Cj0796c is also involved in PG precursor biosynthesis. It is a d-alanyl-d-alanine ligase catalyzing the formation of d-alanyl-d-alanine from two d-alanine residues. The annotation of Cj0796c as a putative hydrolase and its location near PG precursor biosynthetic proteins indicate that it could also be involved in PG biosynthesis. To examine the potential influence of levels of the Cj0796c protein on morphology, the 11168-O 0796 gene was expressed at the rRNA spacer region of 11168-GS expressing 11168-O pgp1. Most transformants showed no change in morphology (Supporting Information Fig. S4). Interestingly, approximately 1/10 transformants produced filamented cells with wild-type helical morphology. These pick-up suppressors easily and lose their filamented phenotype upon passage. Transformation of genomic DNA from filamented, helical 11168-GS strains expressing 11168-O pgp1 resulted in a ratio of filamented cells with wild-type helical morphology to rod-shaped cells of 1/10 seen, as seen with the initial transformation. Preliminary attempts at constructing a 11168-GS deletion mutant were unsuccessful, indicating that Cj0796c may be essential, which is supported by the literature (Metris et al., 2011).

The 11168-GS variant is phenotypically different from 11168-O and a 11168-O-pgp1 mutant

Some of the phenotypes found to be defective in a rod shaped C. jejuni 81-176 mutant were examined in 11168 (Fridrich et al., 2012): motility, biofilms, CFW reactivity and the transition to a coccoid form. As determined previously, the 11168-GS strain was defective for motility in soft agar, displaying 12.1% motility compared with 11168-O. Similar to 81-176 Δpgp1 (Fridrich et al., 2012), 11168-O-Δpgp1 had a slight motility defect at 68.5% of 11168-O motility (Fig. 7A). The level of biofilm production was assessed over 3 days using the crystal violet assay (Fig. 7B). The 11168-O strain produced less biofilm than 81-176. In comparison with 11168-O, biofilm levels of 11168-GS were consistently 2.0-fold higher at Day 1 and then identical to 11168-O at Day 2 and 3. The 11168-O-Δpgp1 strain produced less biofilms than the 11168-O strain only at Day 2. The CFW fluorescence of 11168-O was identical to that of 81-176 (Fig. 7C), both being helical strains with a wild type copy of pgp1. The 81-176 Δpgp1 mutant was hypofluorescent on CFW (as mentioned above; Fridrich et al., 2012), as were the 11168-O Δpgp1 mutant and 11168-GS strains, expected from their straight phenotype resulting from the absence of Pgp1 activity. The 11168-O Δpgp1 mutant could be complemented to wild type CFW fluorescence by insertion of 11168 pgp1 at the rRNA locus (from plasmid pEF84). Introduction of 11168 pgp1 (from plasmid pEF84) into 11168-GS did not complement the morphology defect, or the CFW hypofluorescence.

Campylobacter jejuni transitions to a coccoid form during exposure to environmental stresses such as aging, starvation, suboptimal temperatures, changes in oxygen tension, pH, osmolarity and pressure (reviewed in Svensson et al., 2008; Ikeda and Karlyshev, 2012). For ease of analysis, aging was used as the condition of choice for coccoid formation. Cells were grown at 38°C on solid media under microaerophilic conditions and coccoid formation was monitored by microscopy over time (Fig. 7D). All samples were taken from the center of the plate, as sampling from different areas of the plate showed some variability. The percentage of helical, coccoid, and cells transitioning to the coccoid form was quantified from DIC images (Fig. 7E). After 2 days post-inoculation, approximately 96% of the 11168-O population had transformed from the helical to coccoid form on solid media, while only 26% of 11168-GS was coccoid. The percentage of coccoid bacteria in the 11168-O strain gradually increased to 100% by Day 10 (98% at Day 3, 4, and 5 and up to 100% at Day 10). By Day 3, 11168-GS was 80% coccoid and remained at 80% up to and including Day 10. The delay in coccoid formation observed for 11168-GS was identical to that seen for a Δpgp1 mutant in 81-176 (E. Fridrich and E. Gaynor, manuscript in preparation). Growth curve analysis (Fig. 7F) was used to assess the viability of C. jejuni 11168-O and 11168-GS at long-term timepoints used to examine the transition to the coccoid form in Fig. 7D and E. The viability of 11168-O and 11168-GS was similar at all days despite differences in population morphology, except at Day 2. The drop in 11168-O viability at Day 2 (48h) was observed consistently and was also observed in C. jejuni NCTC 11351 (Martinez-Rodriguez et al., 2004). A maximum cell count is reached upon entering stationary phase at Day 1 (24 h) of 3.0 × 10⁸ for 11168-O (2.0 × 10⁸ for 11351), followed by a decline to 4.2 × 10⁵ (10⁶ for 11351) at Day 2 (48 h), and then an increase at Day 3 (72 h) to 1.1 × 10⁶ (10⁷ for 11351) (Fig. 7F; Martinez-Rodriguez et al., 2004). For 11168-GS, the maximum cell count of 6.4 × 10⁸ is also
reached at Day 1 (24 h) which is followed by a decline to $7.5 \times 10^7$ at Day 2 (48 h) and no further increase in viability beyond the Day 2 timepoint. Strain passage (such as that used to generate 11168-GS from 11168-O) has been noted to result in a reduced rate of viability loss in stationary phase and lack of a re-growth phase.
(Martinez-Rodriguez et al., 2004), explaining the differences in growth curves for 11168-O and 11168-GS.

Discussion

Variations in virulence between C. jejuni strains can at least be partly attributed to the large degree of genetic and resulting phenotypic differences between isolates (van Putten et al., 2009). C. jejuni genetic variation not only occurs between different strains, but also within individual strains, as we have shown in this study. Therefore, C. jejuni is considered to have a partly non-clonal population structure with some clones remaining stable and others showing genetic instability arising during growth. This has been typically attributed to the natural ability of C. jejuni to take up and integrate DNA, intragenomic rearrangements, and phase variation (Wassenaar, 2002). The C. jejuni intra-strain genetic diversity in turn generates phenotypic variation that is hypothesized to increase the ability of some members of the offspring generation to survive varying environmental stresses encountered during transmission under which C. jejuni cannot replicate. The surviving clone(s) would then be able to colonize a new host, and clonal expansion would occur, facilitating bacterial survival. C. jejuni genomic instability may therefore serve as a method of rapid adaptation to different environmental conditions and an alternative strategy to more conventional genetic regulatory mechanisms for sensing the environment and modifying gene expression accordingly. Cell-to-cell heterogeneity, as seen in C. jejuni, has been identified as a widespread microbial phenomenon that can be regulated and is typically observed in genes associated with stress and metabolic functions, and not essential genes (reviewed in Martins and Locke, 2015).

Previously, the C. jejuni genome was shown to encode hypervariable regions or contingency loci that could not be resolved to a consensus sequence during WGS (Parkhill et al., 2000). These hypervariable regions include clusters of genes, such as those involved in the synthesis of the LOS and capsule, flagellar modification and DNA restriction and modification systems (Parkhill et al., 2000). The hypervariable genes contain sequences of short runs of homopolymeric nucleotides (Parkhill et al., 2000) prone to a high rate of slipped-strand mispairing (SSM) during replication. This results in insertions and/or deletions and subsequent frameshift mutations that switch gene function on/off at a rate that is higher than that of spontaneous mutation, creating a subpopulation within the bacterial population. These mutations are heritable and usually reversible. SSM can also occur in promoter regions thereby affecting promoter activity and the expression levels of downstream proteins. SSM is a mechanism of phase variation, a process of generating bacterial population heterogeneity. Phase variation is widespread among bacterial genera of different taxonomic groups and influences bacterial adaptation and virulence by modulating the synthesis of surface exposed molecules such as capsule, flagella, pili, adhesins, and iron-acquisition factors, affecting processes such as motility, biofilm formation and detachment, adhesion, host recognition and evasion of host immune responses (Wisniewski-Dye and Vial, 2008; Darmon and Leach, 2014). Phase variation can also modulate genes involved in metabolism, regulation and DNA restriction-modification systems (Wisniewski-Dye and Vial, 2008; Darmon and Leach, 2014). C. jejuni displays a high rate of phase variation (Bayliss et al., 2012), occurring at poly-G and poly-C tracts (Parkhill et al., 2000), as well as in shorter poly-A tracts (Hendrixson, 2006, 2008) with the frequency of variation depending on the tract length (Bayliss et al., 2012). C. jejuni also lacks a functional DNA mismatch repair (MMR) system responsible for correcting replication errors from single base pair mismatches or SSM arising at homopolymeric tracts, likely adding to the mutation rate seen in C. jejuni and increasing the degree of phase variation (Parkhill et al., 2000; Gaasbeek et al., 2009). Another factor involved in influencing the population heterogeneity and the mutation rate of C. jejuni is the Campylobacter mfd (mutation frequency decline) gene product encoding a transcription-repair coupling factor (Han et al., 2008). In Escherichia coli, Mfd was found to be linked to a “mutation frequency decline” phenotype, preventing the formation of mutations by mediating DNA repair at DNA lesions that stall RNA polymerase during transcription (Savery, 2007; Ganesan et al., 2012). In contrast, C. jejuni Mfd was found to actually promote the appearance of spontaneous mutants (Han et al., 2008). How C. jejuni Mfd affects mutation rates in C. jejuni and whether other proteins are involved in the process is currently unknown.

An increasing number of studies are demonstrating the genomic instability and adaptable mutative properties of C. jejuni. Comparison of the whole genome sequence of a minimally passaged C. jejuni 81-176 strain (81-76/55) (Mohawk et al., 2014) and our laboratory 81-176 strain to the reference 81-76 strain sequenced by The Institute for Genomic Research (TIGR) revealed mutations throughout the genome. These included variations at homopolymeric tracts, SNPs, indels (short insertions or deletions), and substitutions. Non-motile colonial variants of C. jejuni 81-176/55 were isolated by media passaging and were found to result from mutations in the two component regulatory system flgRS regulating flagellar expression [previously reported to be phase variable (Hendrixson, 2006; 2008)].
and from high frequency spontaneous mutations in the motA gene coding for the flagellar motor protein (Mohawk et al., 2014). Another study observed differential motility between defined mutant clones in the flaB gene of C. jejuni M1 (de Vries et al., 2015). Genome sequencing identified second site mutations consisting of SNPs and indels that were responsible for the motility defects observed in some of the flaB mutants (de Vries et al., 2015). This highlights the importance of the examination of several mutant clones and complementation to establishing C. jejuni mutant phenotypes. In this study, exposure to the stress of growth on CFW resulted in selection of C. jejuni 81-176 clones with a straight morphology due to mutations in the pgp1 and pgp2 genes. These mutations included SNPs in pgp1 and pgp2, and SSM in pgp1 at poly-A and -G tracts, as well as at a duplicated sequence. Reversion of the pgp1/+ pgp2/− mutants was not seen by passage on MH. Since straight and helical C. jejuni grow equally well on MH, this is not surprising. Reversion may be seen under conditions favoring a helical morphology, such as during chick colonization.

All 81-176 strains sequenced in this study showed variation in cji81176_1449 and recR gene (cji81176_1279), indicating that these genes are likely to be highly variable in C. jejuni 81-176. The recR gene was also varied in C. jejuni 11168 strains sequenced, but the 11168 homolog of cji81176_1449 was not. The cji81176_1449 gene is unique to Campylobacter species and encodes a hypothetical protein of unknown function. RecR is a recombination mediator protein involved in the homologous recombination pathway of chromosomal DNA repair which is important in maintaining genomic stability. It is conceivable that changes in C. jejuni RecR would affect DNA recombinational repair and could influence the generation of genetic diversity. In H. pylori, RecR functions with RecO to form the RecRO pathway of recombinational repair which was shown to mediate intra-genomic recombination at direct repeat sequences and repair DNA damage in response to oxidative and acid stress (Wang et al., 2011). H. pylori mutants in recR and recO were also defective in host colonization (Wang et al., 2011). The importance of genomic variability in C. jejuni recR and cji81176_1449 remains to be examined.

It is unknown why passage of the wild type strain on CFW favored accumulation of SNPs in pgp1 or pgp2, while passage of Tn mutants on CFW favored frameshift mutations in pgp1 (Table 1). A subsequent Tn mutant screen on CFW likewise identified SNPs in pgp1 and pgp2 in Tn mutants unlinked to the Tn insertion (J. Vermeulen, E. Fridrich, and E. Gaynor, unpublished). All frameshift mutants resulted in truncated versions of Pgp1 (Table 1) thereby abrogating function. The point mutations in pgp1 and pgp2 also produced non-functional proteins. The Pgp1 S402F and V175F substitutions in 81-176 rod shaped isolates 33-1 and 12-1, respectively, were not in active site residues of Pgp1, as determined by conserved domain analysis and comparison with the crystal structure of the H. pylori Csd4 homolog (Chan et al., 2015), but are required for Pgp1 activity. Introducing a Phe preserves the hydrophobic nature of the wild type amino acids, but is also more bulky and may push the protein apart. The V175F mutation is in the N-terminal carboxypeptidase domain located on a helix away from the active site (Chan et al., 2015), but may have an allosteric effect on activity. The S402F mutation is in a semi-flexible surface loop region in the last domain (domain 3) that is required for activity and predicted to be involved in protein–protein or protein–PG interactions (Chan et al., 2015). The point mutation in Pgp2 resulted in a G131C. There is no crystal structure available for Pgp2, but the amino acid change is in the conserved co-catalytic domain YkuD as predicted from conserved domain analysis (Supporting Information Fig. S3). Changing a Gly to a Cys could cause considerable changes in protein structure that could affect activity such as: a reduction in flexibility of that region which may be required for function or the formation of disulfide bonds between Cys residues resulting in a loss of protein stability and aggregation. All mutated copies of pgp1 and pgp2 could be complemented with a wild type copy of the gene.

The nature of the stress imposed by CFW on C. jejuni is unclear as well as why straight mutants are more prevalent after growth on CFW. Helical and straight strains both grow equally well in media with and without CFW and have a similar MIC to CFW (data not shown), so CFW-induced selection of straight variants is not due to differences in growth rate. Since straight mutants all display a dim phenotype on CFW and therefore fail to bind CFW, this must be somehow advantageous to the cell. CFW does bind to the β 1-4 linkages of the PG backbone (E. Fridrich and E. Gaynor, unpublished). CFW binds equally well to isolated PG from wild type and Δpgp1 (E. Fridrich and E. Gaynor, unpublished); however, this may not be the case with cellular PG. The differences in the length of the PG amino acid side chains in a straight mutant in comparison with the helical strain may alter the accessibility of CFW to the PG backbone, limiting CFW binding and the deleterious effects of CFW on the cell.

The selection of straight variants after passage on CFW can be attributed to either: (1) the presence of straight cells in the original C. jejuni 81-176 population prior to exposure to CFW, or (2) the development of straight variants after exposure to CFW. In the first scenario, examination of the cellular morphology of a population of C. jejuni 81-176 should show the presence of
straight cells among the helical cells even at a very low percentage. This has not been observed in our laboratory at any stage of C. jejuni 81-176 growth (E. Frirdich and E.C. Gaynor, unpublished). In the second scenario, variation in pgp1 and pgp2 would occur likely by an increase in the level of phase variation (as in the pgp1 frameshift mutations) and mutation rate (to generate SNPs, as in 12-1, 25-1, and 33-1) after exposure to CFW. Environmental or intracellular host factors (such as temperature, pH, nutrient availability, oxygen levels, and the availability of iron) have been shown to affect the rate and timing of phase variation in other bacteria, thereby controlling the generation of variants and increasing the appearance of subpopulations that are more likely to successfully survive the stress (van der Woude and Broadbent, 2011; Darmon and Leach, 2014). WGS of strains passaged on CFW (transposon mutants dim111 and dim128, and 81-176 variants 12-1, 12-10, 25-1, 25-6, 33-1, and 33-2; Supporting Information Table S3) did not display any genomic changes unique to CFW exposure when compared with a non-CFW passed wild-type 81-176 strain. In addition, initial proteomic studies with 81-176 grown with and without CFW showed no observable differences in protein abundance (N.E. Scott, E. Frirdich, J. Vermeulen, and E.C. Gaynor, unpubl. obs.). Unlike the CFW-passaged 81-176 variants, the dim mutants selected for on CFW had an increased total number of genomic changes in comparison with non-CFW passed 81-176 (78% for dim111, and 66% for dim128) (Supporting Information Table S3). The process of Tn mutagenesis or maintenance of the Tn may have induced additional variation in these strains.

Our current hypothesis is that the binding of CFW to C. jejuni PG results in envelope stress that triggers the upregulation of phase variation and increases mutation rates in C. jejuni, thereby resulting in pgp1 and pgp2 mutations that produce C. jejuni variants more tolerant to the effects of CFW. To further clarify this we need to determine the nature of the stress posed by CFW on C. jejuni, how envelope stress is recognized in C. jejuni, and whether phase variation is regulated in C. jejuni. Passaging of C. jejuni NCTC 11168 through mice (Jerome et al., 2011), chickens (Bayliss et al., 2012) and through humans (after accidental infection) (Revez et al., 2013; Thomas et al., 2014), resulted in the selection of variants often displaying increased virulence. The variants were primarily at homopolymeric tracts in phase variable contingency genes, although one study did identify the presence of SNPs in the genome (Thomas et al., 2014). The variants isolated after human infection were distinct from those isolated from animal models (Revez et al., 2013) and a human infection isolate when inoculated into mice underwent additional genetic variation (Thomas et al., 2014). This emphasizes the important role that C. jejuni genetic heterogeneity plays in adaptation to different host environments. Interestingly, substantial genetic changes only occurred during host passage and not during growth in vitro (Bayliss et al., 2012; Kim et al., 2012; Thomas et al., 2014), supporting the idea that phase variation may be regulated in C. jejuni.

Sequence analysis showed that the nature of the straight morphology of the 11168-GS strain characterized previously in our laboratory (Gaynor et al., 2004) resulted from a frameshift mutation in pgp1. This frameshift mutation occurred due to the loss of a G nucleotide at a –GG– sequence (Supporting Information Fig. S1). Whether this occurred due to SSM is difficult to determine, as a rough threshold of four repeat units has been proposed as the minimum number resulting in mutation by SSM (Zhou et al., 2014). The straight 11168-GS variant of the helical 11168-O strain is thought to have arisen due to laboratory passage (Gaynor et al., 2004), although repeated passage of 11168-O (up to 25 times) in our laboratory has not resulted in the appearance of a straight 11168-O variant (E. Frirdich and E.C. Gaynor, unpubl. obs.). The 11168-GS strain may therefore have been subjected to additional stresses during laboratory passage that resulted in the selection of this straight variant. With the now relative ease of WGS, several complete genome sequences of NCTC 11168 are currently available and have been used to identify genetic changes affecting virulence by comparing more virulent strains to the avirulent 11168-GS strain (Jerome et al., 2011; Revez et al., 2012, 2013; Cooper et al., 2013). None of the genome sequenced 11168 strains [including the 11168-GS deposited in GenBank by Parkhill et al. (2000)] have mutations in pgp1, indicating that the 11168-GS strain used by our laboratory may be the only variant with a morphology defect. This further highlights the fact that NCTC 11168 strains vary greatly between laboratories, making experimental comparisons extremely difficult.

The similar muropeptide profiles between the 11168-GS strain and a 11168-O-Δpgp1 mutant indicate that the frameshift mutation in 11168-GS pgp1 resulted in an inactive version of the Pgp1 protein and that this loss of Pgp1 activity is responsible for the straight morphology of the 11168-GS strain. However, unlike the 11168-O-Δpgp1 mutant and the 11168-M strain also harboring a frameshift mutation in pgp1, introduction of a wild type copy of pgp1 was unable to restore the wild type helical morphology to 11168-GS and produced cells with a very minimal helical pitch. This could result from several factors. (1) Differences in the levels of Pgp1 [increases and decreases in Pgp1 levels affect morphology (Frirdich et al., 2012)]. The level of pgp1 expression was higher.
in 11168-GS and 11168-GS overexpressing 11168-O pgp1 than the 11168-O strain. The levels of pgp1 in 11168-O were similar to those of the rod-shaped 11168-M and 11168-O-Δpgp1 strains, as well as the 11168-M and 11168-O-Δpgp1 strains overexpressing 11168-O pgp1 that could be complemented to a wild-type helical morphology. Therefore, the higher level of Pgp1 in 11168-GS strains could be preventing proper shape complementation. (2) Mutation in a gene responsible for dictating the degree of helical pitch (candidates are summarized in Supporting Information Table S6). Of the ones examined, cell shape determinant mreB (cJ0276), hydrolase cj1305c and the glycopeptide cj0455c, none were able to restore wild type helicity. Lastly, (3) a change in abundance of proteins affecting the cell wall and cell shape (Scott et al., 2014). Several proteins affecting peptidoglycan biosynthesis and thereby morphology were present in lower amounts in 11168-GS in comparison with 11168-O: the penicillin binding-proteins PbpA and PbpC, the tubulin-like cell division protein FtsZ (previously shown to affect cell shape (Varma and Young, 2004)), the amino acid ligase MurF involved in UDP-MurNAc pentapeptide precursor synthesis (Scott et al., 2014), and the putative hydrolase Cj0796/ Cj81176_0817 (although its role in cell morphology remains to be established). It is possible that the levels of these proteins could affect morphology.

Despite the similar cellular morphology and PG muropeptide profiles between our 11168-GS strain and a 11168-O-Δpgp1 mutant, other phenotypes associated with a deletion of pgp1 (such as motility and biofilm formation; (Frirdich et al., 2012)] that are altered in comparison with wild type 11168-O also differ between 11168-O-Δpgp1 and our 11168-GS strain (Fig. 7). This can be attributed to the numerous other genomic changes (Gaynor et al., 2004) and differences in protein abundance (Scott et al., 2014) between 11168-GS and 11168-O.

It is now well accepted that C. jejuni isolates consist of a dynamic population of varying genotypes and therefore phenotypes that undergo diversification during exposure to stress and different host environments. This population heterogeneity allows for rapid adaptation and survival under new conditions. The appearance of rod shaped variants, such as those arising from exposure to CFW described in this study, our laboratory 11168-GS strain (Gaynor et al., 2004), those generated by passage through chick embryos (Field et al., 1991, 1993) and chickens (Hanel et al., 2009), and those that appeared in some flagellar mutants (Wassenaar et al., 1991; Matz et al., 2002; Fernando et al., 2007) that are unlinked to the flagellar mutation indicate that the ability of C. jejuni to alter its shape from helical to straight provides a selective advantage to C. jejuni under certain stress conditions. The exact nature of this stress remains to be determined. The pgp1 gene can now be added to the repertoire of phase variable genes in C. jejuni. Loss of pgp1 and the associated changes in PG structure affect numerous C. jejuni pathogenic attributes and recognition by host receptors, so it is not surprising that changes in this gene and in morphology would affect how C. jejuni interacts with its environment.

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study and their construction are described in the Supplemental files (Supporting Information Tables S1 and S2). C. jejuni strains were grown at 38°C in Mueller-Hinton (MH; Oxoid) broth or on 8.5% (w/v) agar plates supplemented with vancomycin (V; 10 μg/ml) and trimethoprim (T; 5 μg/ml) (unless otherwise indicated) under microaerobic and capnophilic conditions (6% O2, 12% CO2) in a Sanyo tri-gas incubator for plates or using the Oxoid CampyGen system for broth cultures. Growth media was supplemented with chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), and apramycin (60 μg/ml) where appropriate. E. coli strains used for plasmid construction were grown at 38°C in Luria-Bertani (LB; Sigma) broth or 7.5% agar (w/v) agar plates and supplemented with ampicillin (100 μg/ml), chloramphenicol (15 μg/ml), or kanamycin (25 μg/ml), as necessary.

For growth analysis and DNA analysis, C. jejuni strains were streaked from fresh plate cultures and grown for 5–7 h. They were harvested in MH-TV broth and inoculated at an OD600 of 0.002 into MH-TV broth and grown shaking for 18 h. Strains were subcultured to an OD600 of 0.002 and samples were taken at various time points for CFU and microscopic analysis.

Microscopy

For visualization under bright field or differential interference contrast (DIC) microscopy, 1 μl of a broth or plate culture was immobilized on a thin 1% agar (w/v) in H2O2 slab and overlaid with a cover slip. Images were captured with a Nikon Eclipse TE2000-U microscope equipped with 100× objective and a Hamamatsu Orca camera system. Quantification of the percentage of helical, coccoid, and cells transitioning to the coccoid form in the DIC images was carried out by counting the number of each using ImageJ software (NIH). At least three separate fields of view of approximately 150–200 bacteria were counted for each strain at each time point and this was repeated for three separate cultures.

Whole-genome sequencing

Genomic DNA for all experiments was harvested via Wizard genomic DNA purification (Promega). Illumina libraries were prepared using the KAPA Low-Throughput Library
Phenotypic assays were carried out with strains grown in shaking MH-TV broth for 18 h. For motility, cultures were diluted to an OD$_{600}$ of 0.2 in MH-TV and 2 μl was point inoculated into MH-TV plates containing 0.4% agar. Plates were incubated for 20 h and the halo diameter was measured. Biofilm formation was assayed using crystal violet staining and CFW fluorescence was described previously (Fridrich et al., 2012).

**Peptidoglycan isolation and muropeptide analysis**

PG isolation, muropeptide generation, separation by HPLC and identification was carried out as described previously (Fridrich et al., 2012; 2014). Briefly, *C. jejuni* strains were passaged once from frozen stocks, passaged to 20-25 MH plates and grown for 18–20 h. Cells were collected into cold MH broth by scraping to give a final OD of 200–600, harvested by centrifugation at 8000 × g for 15 min and then resuspended in 6 ml ice cold H$_2$O. Cells were lysed by dropwise addition to 6 ml 8% SDS boiling under reflux. PG was purified from the cell lysate, digested with the muramidase cellosyl (kindly provided by Hoechst, Frankfurt, Germany), and the resulting muropeptides were reduced with sodium borohydride and separated by HPLC as described (Glauner, 1988). Muropeptide structures were assigned based on (i) comparison with retention times of known muropeptides from *C. jejuni* (Fridrich et al., 2012) and (ii) by mass spectrometry (MS). For MS analysis, muropeptide fractions were collected, concentrated in a SpeedVac, acidified by 1% trifluoroacetic acid, and analyzed by offline electrospray mass spectrometry on a Finnigan LTQ-FT mass spectrometer (ThermoElectron, Bremen, Germany) at the Newcastle University Pinnacle facility as described (Bui et al., 2009).

**Real-time quantitative PCR**

*Campylobacter jejuni* strains were grown as described above. After 24 h of growth from a culture inoculated at an OD$_{600}$ of 0.002, RNA was isolated and cDNA was synthesized as described (Svensson et al., 2009). Absence of genomic DNA within RNA samples was confirmed by PCR and by including a no reverse transcriptase control in the cDNA reactions. Quantitative PCR was performed using primers designed to *pgp1* (1344-SYBR-F2/-R2; Supporting Information Table S2). Reactions were set up with IQ SYBR green Supermix (Bio-Rad) and performed with a CFX96 real-time PCR detection system (Bio-Rad) as directed by the manufacturer. Reactions were performed in triplicate on three independent samples. Relative quantification was carried out by normalizing against unit mass (10 ng cDNA) and relative expression of *pgp1* was calculated by the ΔC$_T$ method. A reference gene was not used for relative quantification as the amplification efficiency calculated from a standard curve with various template preparations and different sets of primers to the commonly used *C. jejuni* reference genes *rrs* (16S rRNA; Hyttiläinen et al., 2012) or *rpoA* (α-subunit of DNA-directed RNA polymerase; Ritz et al., 2009) was too low (<90%).

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References


**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.