Accumulation of Peptidoglycan O-Acetylation Leads to Altered Cell Wall Biochemistry and Negatively Impacts Pathogenesis Factors of Campylobacter jejuni*‡

Reuben Ha1,2, Emilia Frirdich8, David Sychantha9, Jacob Biboy1, Michael E. Taveirne2, Jeremiah G. Johnson3, Victor J. DiRita4,5, Waldemar Vollmer6, Anthony J. Clarke5, and Erin C. Gaynor15

From the 4 Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, the 1 Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada, the 6 Centre for Cellular Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne NE2 4AX, United Kingdom, and the 3 Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109

Campylobacter jejuni is a leading cause of bacterial gastroenteritis in the developed world. Despite its prevalence, its mechanisms of pathogenesis are poorly understood. Peptidoglycan (PG) is important for proper cell growth, characterized by acetylation of the C6 hydroxyl group of N-acetylmuramic acid in the PG glycan backbone. The OAP gene cluster consists of a PG O-acetyltransferase A (patA) for translocation of acetate into the periplasm, a PG O-acetyltansferase B (patB) for O-acetylation, and an O-acetylpeptidoglycan esterase (ape1) for de-O-acetylation. In this study, reduced OAP in ΔpatA and ΔpatB had minimal impact on C. jejuni growth and fitness under the conditions tested. However, accumulation of OAP in Δape1 resulted in marked differences in PG biochemistry, including O-acetylation, anhydromuropeptide levels, and changes not expected to result directly from Ape1 activity. This suggests that OAP may be a form of substrate level regulation in PG biosynthesis. Ape1 acetylerase activity was confirmed in vitro using p-nitrophenyl acetate and O-acetylated PG as substrates. In addition, Δape1 exhibited defects in pathogenesis-associated phenotypes, including cell shape, motility, biofilm formation, cell surface hydrophobicity, and sodium deoxycholate sensitivity. Δape1 was also impaired for chick colonization and adhesion, invasion, intracellular survival, and induction of IL-8 production in INT407 cells in vitro. The importance of Ape1 in C. jejuni biology makes it a good candidate as an antimicrobial target.

Campylobacter jejuni is a leading bacterial cause of foodborne gastroenteritis in the developed world and the most common infectious antecedent to the autoimmune acute polynuropathy Guillain-Barré syndrome (1, 2). As a commensal of the avian gut, it is a prevalent contaminant of uncooked poultry (3). Because of its high incidence rate, the costs of C. jejuni infection are a significant socioeconomic burden, making it both a health care concern and an economic issue (4). In addition, C. jejuni has been exhibiting alarming increases in resistance to ciprofloxacin and erythromycin, the most commonly used antibiotics for treatment of C. jejuni infection (5). Despite its prevalence, relatively little is known about C. jejuni pathogenesis in humans. Traditional virulence factors present in other gastrointestinal pathogens are either absent (i.e. dedicated type III secretion systems) or limited (C. jejuni possesses some stress-response elements such as the stringent response modulator SpO, but it lacks several hallmark stress-response elements like RpoS and RpoE), or their role in pathogenicity is debated (i.e. the cytolysogenic distending toxin and a putative type IV secretion system on the pVir plasmid) (6–11). However, factors considered to be fundamental aspects of bacterial physiology such as metabolism, stress response, and cell shape are hypothesized to play important roles in C. jejuni pathogenesis (12, 13).

The peptidoglycan (PG) sacculus is a heteropolymer of the bacterial cell wall composed of alternating β-1-4 N-acetylglyc
Role of C. jejuni Peptidoglycan O-Acetylation

cosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues cross-linked to adjacent strands by peptides bound to the MurNAc residue. It is responsible for providing structural strength to the cell, enabling it to resist changes in osmotic pressure, and for maintenance of cell shape (14–16). The cork-screw motility of C. jejuni generated by its helical shape and polar flagella is thought to be important in enhancing its ability to move through viscous media, such as the mucus layer of the gastrointestinal tract (14, 17). Deletion of PG hydrolase enzymes Pgp1 and Pgp2 in C. jejuni has led to a change in morphology from helical to straight with accompanying defects in traits associated with pathogenesis, including motility in soft agar, biofilm formation, and chick colonization. PG isolated from Δpgp1 and Δpgp2 also exhibited altered stimulation of host cell NOD receptors, and Δpgp1 elicits an enhanced pro-inflammatory IL-8 response from INT407 epithelial cells upon infection (18, 19). Changes in PG biosynthesis and composition as well as the release of PG products have long been known to affect physiological and pathogenic properties of many bacterial species (20), including Listeria monocytogenes (21, 22), Helicobacter pylori (23), and Streptococcus pneumoniae (24). Current research continues to support this concept (15). Some recent studies have shown that changes in morphology and PG structure in Mycobacterium tuberculosis affect its physiology and virulence in mice (25); changes in morphology in Proteus mirabilis affect its swarming motility (26); and changes in morphology in Helicobacter pylori alter its motility and colonization potential (27, 28).

Understanding PG biosynthetic mechanisms in C. jejuni may prove advantageous to the development of new antimicrobials. It has been suggested that O-acetylation of peptidoglycan (OAP) machinery may be an attractive target (29–33). OAP occurs in both Gram-positive and Gram-negative bacteria and is characterized by the addition of an acetyl group to the C6 hydroxyl group of MurNAc in the PG glycan backbone (Fig. 1A). This modification confers resistance to lysozyme (34, 35), which cleaves β-1,4-glycosidic bonds between MurNAc and GlcNAc (36). Despite the intrinsic resistance to lysozyme provided by the outer membrane of Gram-negative bacteria, lysozyme resistance was shown to be important in H. pylori using mutants defective in OAP addition and similar glycan modifications (37). These strains showed increased susceptibility to physiologically relevant concentrations of lysozyme in the presence of the host lactoferrin, which has membrane permeabilization properties (38, 39). O-Acetylated gonococcal PG is implicated in the development of arthritic symptoms and is hypothesized to be attributable to increased PG hydrolase resistance leading to large fragments of circulating PG (40). In addition, OAP is believed to be involved in the regulation of PG turnover by inhibiting endogenous lytic transglycosylase (LT) activity. LTs require an unmodified MurNAc C6 hydroxyl moiety to cleave β-1,4-glycosidic bonds between MurNAc and GlcNAc, generating anhydromuropeptides (anhMPs). LTs are therefore important for generating insertion sites for newly synthesized muropeptides during cell growth and division (41).

The OAP gene cluster was initially identified in Neisseria gonorrhoeae and was found to be responsible for OAP in many Gram-negative pathogens (42). It consists of a putative trans-membrane protein, PG O-acetyltransferase A (PatA) predicted to be involved in the translocation of acetyl moieties from a cytoplasmic source into the periplasm, a periplasmic transferase, PG O-acetyltransferase B responsible for O-acetylation of MurNAc, and a periplasmic O-acetylpeptidoglycan esterase (Ape1) for MurNAc de-O-acetylation (Fig. 1A and B) (42–44). Since their discovery, PatB and Ape1 from N. gonorrhoeae and Neisseria meningitidis have been well characterized biochemically, including descriptions of the catalytic residues, mechanism of enzyme activity, and substrate specificity (30, 32, 43, 45). The availability of a recently solved crystal structure for N. meningitidis Ape1, recently identified Ape1 inhibitors, and studies showing reduced septicemia in mice infected with N. meningitidis ape1 mutants lend support for the application of Ape1 as an antimicrobial target (31, 33, 46).

C. jejuni encodes previously unstudied homologs of the OAP genes patA, patB, and ape1. In this work, the roles of these genes in PG O-acetylation and overall PG biosynthesis, as well as biological and pathogenic attributes were assessed via construction of strains deleted for each or all of these genes followed by biochemical and phenotypic analyses. Each mutant exhibited changes in PG O-acetylation consistent with predicted gene function products. The accumulation of O-acetylated PG was found to be detrimental to C. jejuni fitness, whereas diminished O-acetylation had little to no effect. Δape1 exhibited defects in PG muropeptide composition, cell morphology, pathogenic attributes, and host-pathogen interactions, whereas ΔpatA, ΔpatB, and Δoap mutants exhibited no or, in rare cases, only minimal defects for these phenotypes.

Results

C. jejuni OAP Genes Were Identified by BLAST and Mutant and Complemented Strains Were Generated—The OAP gene cluster was identified in C. jejuni 81-176 wild type by BLAST analysis using the N. gonorrhoeae OAP gene sequences. The loci identified were cjj81176_0640, cjj81176_0639, and cjj81176_0638 for patA, patB, and ape1, respectively (Fig. 1B). Amino acid sequence identity and sequence similarity for these genes were 35/53%, 39/57%, and 26/44% identity/similarity to N. gonorrhoeae patA, patB, and ape1, respectively.

To investigate the role of OAP in C. jejuni, the patA, patB, and ape1 homologs, as well as the entire gene cluster, were inactivated by deletion-insertion mutagenesis with the non-polar KmR cassette (aphA-3) from pUC18K-2 lacking a transcriptional termination site (Fig. 1C; supplemental text S1) (47). Complementation was achieved using the pRRC integration vector (48). For complementation, the coding region of each OAP gene plus upstream sequence containing the ribosomal binding site was inserted into the genome of the corresponding mutant at ribosomal intergenic regions along with a CmR cassette (the Δape1 complemented strain, designated Δape1C, is shown in Fig. 1D as an example). Expression of the wild-type genes at the rRNA site was driven from the promoter of the CmR cassette.

Growth rate experiments performed on all mutant strains indicated no significant difference in growth rates in broth cultures up to 36 h (data not shown) with the exception of Δape1C, which grew at a slower rate (although this slower growth rate
Role of C. jejuni Peptidoglycan O-Acetylation

Respond to the question: What did not affect the ability of Δape1C to complement Δape1 phenotypes? Differences were observed in the long term survival properties of Δape1 with more modest differences exhibited by ΔpatA, ΔpatB, Δoap, and Δape1C. At 48 h, a 1.0-log increase in recovery was observed for Δape1, but recovery fell at 72 h by 0.8-log relative to wild type. At 48 h, ΔpatA and ΔpatB exhibited a 0.3-log increase, and Δoap showed a 0.4-log increase in recovery, relative to wild type. The recovery of all three OAP-deficient mutants was 0.3-log lower than wild type at 72 h. Δape1C exhibited a 0.8-log increase in recovery at 48 h and a 0.3-log decrease at 72 h relative to wild type (data not shown).

O-Acetylation Levels of Purified PG from Deletion Mutants Reflect the Putative Functions of the C. jejuni OAP Gene Cluster—To determine whether the C. jejuni OAP gene homologs were involved in OAP, PG O-acetylation levels were determined for the mutants of the three putative OAP genes (ΔpatA, ΔpatB, and Δape1) and for the mutant lacking the entire cluster (Δoap) (Fig. 1C). PG was isolated from strains using an established protocol that minimizes spontaneous O-linked acetate hydrolysis and was assessed for OAP levels by quantifying released acetate and MurNAc content (49, 50).

The O-acetylation level for the wild-type strain 81-176 was determined to be 12.5 ± 0.71% relative to MurNAc content. O-Acetylation levels among the mutants varied according to their predicted function (Table 1). Deletion of patA and patB resulted in a reduction in O-acetylation levels at 2.45 ± 0.14 and 3.05 ± 0.22% relative to MurNAc content, respectively. Deletion of the entire gene cluster in Δoap resulted in a decrease in O-acetylation levels to 2.10 ± 0.18%, similar to that of ΔpatA and ΔpatB. Previous studies using Escherichia coli (which lacks PG O-acetyl groups) showed undetectable levels of acetate using identical methods (50, 51). This suggests that patA/B contributes to PG O-acetylation in C. jejuni, but their absence is insufficient to abolish OAP. Deletion of ape1 led to an increase in O-acetylation to 35.6 ± 2.25% relative to total MurNAc content. These results are in accordance with the functions described for homologs in N. gonorrhoeae and N. meningitidis (30, 42–44). O-Acetylation levels were restored to wild-type levels in the Δape1 complement (11.78 ± 0.52%). Analysis of the O-acetylation levels for ΔpatA and ΔpatB complements were not performed as, unlike the Δape1 mutant, phenotypic differences between these mutants, Δoap and wild type, were in almost every case not statistically significant or were minimal (see below).

C. jejuni OAP Mutants Exhibit Altered PG Muropeptide Profiles, with Δape1 Displaying the Most Dramatic Changes from Wild Type—O-Acetylation has been described as a PG maturative event occurring after transglycosylation and transpeptidation (30). Cleavage of PG by bacterial LTs is inhibited by PG O-acetylation. Thus, O-acetylation may impact PG maturation

TABLE 1

| Strain | % O-acetylation\(^a\) (mean ± S.D.) |
|--------|----------------------------------|
| 81-176 | 12.5 ± 0.71                      |
| Δape1  | 35.6 ± 2.25                      |
| Δape1C | 11.8 ± 0.52                      |
| ΔpatB  | 3.05 ± 0.22                      |
| ΔpatA  | 2.45 ± 0.14                      |
| Δoap   | 2.10 ± 0.18                      |

\(^a\) Results shown are of one representative biological replicate measured in triplicate ± S.D.
Role of C. jejuni Peptidoglycan O-Acetylation

FIGURE 2. HPLC elution profile of C. jejuni muropeptides and proposed muropeptide structures. Purified PG 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 wild-type strain 81-176 (A and E), Δape1 (B), ΔpatB (C), ΔpatA (D), Δape1Δ (F), and Δoap (G). Muropeptide profiles were generated in two sets of experiments indicated by (1) for Sample Set 1 and (2) for Sample Set 2. The muropeptide structure represented by each peak was determined previously by mass spectroscopy (18), and proposed muropeptide structures of each peak corresponding to the peak number in the chromatogram are shown in H. The summary of the muropeptide composition is shown in Table 3. G, N-acetylgulosamine; M, reduced N-acetylmuramic acid; Δ-Ala, Δ-alanine; Δ-Dap, Δ-isoglutamic acid; meso-Dap, meso-diaminopimelic acid; α-Ala, α-alanine Ac, O-acetyl groups at MurNAc C6 position; Anh, 1,6-anhydro group of MurNAc; *, it is not known on which MurNAc residue the modification occurs.

events in C. jejuni, affecting aspects such as muropeptide profiles and glycan chain length (52). To investigate this, PG was isolated from wild type and each of the mutant and complemented strains, and the muropeptide composition was determined. HPLCs are shown in Fig. 2. Raw data (relative abundance of each muropeptide) and summarized muropeptide profiles can be found in Tables 2 and 3, respectively.

The method used for muropeptide analysis results in the loss of some of the PG O-acetylated groups (due to the alkaline conditions for NaBH4 reduction resulting in base-catalyzed hydrolysis of the O-linked acetate) and is thus less precise at determining O-acetylation levels than the methodology used above. Nonetheless, similar trends were observed, further supporting gene product function. PG O-acetylation levels were reduced in ΔpatA, ΔpatB, and Δoap and increased in Δape1 relative to wild-type 81-176. Monomeric O-acetylated tetrapeptide species and O-acetylated tetra-tetra dimeric species were absent in ΔpatB, ΔpatA, and Δoap. The abundance of all detectable O-acetylated muropeptide species was increased in Δape1.

Δape1 exhibited a large decrease in total anhMP species and an increased average glycan chain length compared with wild type, similar to observations made in N. meningitidis (33), and is consistent with the observed O-acetylation levels, as de-O-acetylation must precede LT activity. Conversely, in the absence of patA or patB, the relative abundance of anhMP species did not vary strongly from wild type.

Changes were also observed in other muropeptide species between wild type and ΔpatA, ΔpatB, Δoap, and Δape1. The
Role of C. jejuni Peptidoglycan O-Acetylation

TABLE 2
Muropeptide composition of C. jejuni wild-type 81-176, Δape1, ΔpatB, and Δpata, Δape1C, ΔpatB, ΔpataC, and Δoap showing relative abundance of muropeptides corresponding to peaks in HPLCs (Fig. 2).

| Peaks | Muropeptide | 81-176 | Δape1 | ΔpatB | Δpata | 81-176 | Δape1C | ΔpatB | ΔpataC | Δoap |
|-------|-------------|--------|-------|-------|-------|--------|-------|-------|-------|------|
| 1     | Tri         | 1.2    | 1.3   | 1.4   | 1.5   | 1.6    | 1.7   | 1.8   | 1.9   | 2.0  |
| 2     | Tetra-Gly-4 | 1.1    | 1.2   | 1.3   | 1.4   | 1.5    | 1.6   | 1.7   | 1.8   | 1.9  |
| 3     | Tetra-Ac    | 1.0    | 1.1   | 1.2   | 1.3   | 1.4    | 1.5   | 1.6   | 1.7   | 1.8  |
| 4     | Tetra-Gly-5 | 0.9    | 1.0   | 1.1   | 1.2   | 1.3    | 1.4   | 1.5   | 1.6   | 1.7  |
| 5     | Tetra-Ac    | 0.8    | 0.9   | 1.0   | 1.1   | 1.2    | 1.3   | 1.4   | 1.5   | 1.6  |
| 6     | Tetra-Ac    | 0.7    | 0.8   | 0.9   | 1.0   | 1.1    | 1.2   | 1.3   | 1.4   | 1.5  |
| 7     | Di          | 0.6    | 0.7   | 0.8   | 0.9   | 1.0    | 1.1   | 1.2   | 1.3   | 1.4  |

% Peak numbers correspond to those from HPLCs in Fig. 2. Muropeptides are named according to Glauner et al. (88) and are depicted in Fig. 2H. Di, disaccharide dipeptide; Tetra, disaccharide tetrapeptide; Penta, disaccharide pentapeptide; Muropeptides 1–7 are monomeric, and muropeptides 8–20 are dimers and trimers connected by peptide cross-links. Modifications: Gly, glycine in position 5 of a peptide side chain; Ac, O-acetyl groups at the C-6 hydroxyl group of MurNAc; Anh, 1,6-anhydro-N-acetylmuramic acid.

* Peak was previously unidentified in Sample Set #1.

The total abundance does not add up to 100% due to the presence of peaks for which a structure has not been assigned.

TABLE 3
Summary of PG muropeptide composition for C. jejuni 81-176, Δape1, ΔpatB, Δpata, Δape1C, ΔpatB, ΔpataC, and Δoap.

| Muropeptide species | Sample Set #1 | Sample Set #2 | Sample Set #2 |
|---------------------|---------------|---------------|---------------|
|                     | 81-176 | Δape1 | ΔpatB | Δpata | 81-176 | Δape1C | ΔpatB | ΔpataC | Δoap |
| Monomers (total)     | 43.5   | 49.1  | 44.9  | 41.7  | 42.2  | 41.5  | 39.6  | 41.2  | 40.0  |
| Dipeptide           | 17.8   | 11.0* | 18.0  | 15.0  | 13.6  | 11.0  | 11.2  | 17.2  | 16.4  |
| Tripeptide          | 6.7    | 16.4* | 8.5   | 10.2* | 10.1  | 11.1  | 9.7   | 6.6*  | 6.7*  |
| Tetrapeptide        | 18.3   | 20.9  | 17.6  | 15.8  | 17.2  | 18.1  | 17.1  | 16.1  | 15.5  |
| Pentapeptides-Gly   | 1.2    | 0.9*  | 0.7   | 0.7   | 0.8   | 1.0   | 1.3   | 0.9*  | 1.0   |
| O-Acetylated         | 1.4    | 1.0   | 0.8*  | 0.3*  | 1.9   | 2.4   | 1.7   | 1.1*  | 0.8*  |
| Dimers (total)       | 47.0   | 47.0  | 47.6  | 49.4  | 48.8  | 49.4  | 49.5  | 48.5  | 48.1  |
| TetraTri            | 16.8   | 14.2  | 18.4  | 15.5  | 16.0  | 15.5  | 15.2  | 15.7  | 15.2  |
| TetraTetra          | 30.5   | 25.6  | 31.0  | 33.0  | 32.0  | 33.2  | 32.0  | 30.7  | 15.7  |
| TetraPentaGly       | 0.4    | 0.9*  | 0.6*  | 1.0*  | 0.8   | 0.9   | 1.1*  | 0.8   | 1.2*  |
| Anhydro-Dimers      | 15.0   | 8.2*  | 12.7  | 11.8  | 11.3  | 12.1  | 11.8  | 12.6  | 12.4  |
| O-Acetylated         | 2.2    | 4.9*  | 1.7   | 3.2   | 3.7   | 2.3   | 0.4*  | 0*    | 0*    |
| Trimers (total)      | 8.8    | 10.2  | 7.5   | 8.8   | 7.8   | 8.1   | 9.5   | 8.9   | 10.2*  |
| TetraTetraTri       | 0.9    | 0.5*  | 1.0   | 1.5*  | 1.0   | 0.9   | 1.3*  | 1.3*  | 1.4*  |
| TetraTetraTetra     | 8.0    | 9.7   | 6.5   | 7.3   | 6.8   | 7.2   | 8.2   | 7.6   | 8.8   |
| Dipeptide           | 17.8   | 11.0* | 18.0  | 15.0  | 13.6  | 11.0  | 11.2  | 17.2  | 16.4  |
| Tripeptide          | 15.4   | 23.6* | 18.0  | 19.9  | 18.4  | 19.1  | 17.7  | 14.9  | 14.7  |
| Tetrapeptides (total)| 65.0  | 64.0  | 62.9  | 63.9  | 65.1  | 67.1  | 67.6  | 64.8  | 65.1  |
| Pentapeptides (total)| 0.8   | 1.3*  | 1.0   | 1.2*  | 1.2   | 1.5   | 1.9   | 1.3   | 1.6*  |
| O-Acetylated         | 2.1    | 10.4* | 1.2*  | 0.3*  | 3.5   | 4.3   | 2.9   | 1.3*  | 0.8*  |
| Anhydromuramopeptides| 8.3    | 4.8*  | 7.8   | 7.5   | 7.7   | 7.4   | 7.9   | 8.3   | 8.6   |
| Average chain length | 12.0   | 20.8* | 12.8  | 13.3  | 13.0  | 13.5  | 12.6  | 12.0  | 11.6  |
| Degree of cross-link | 29.7   | 27.2  | 28.8  | 30.6  | 29.6  | 30.1  | 31.1  | 30.2  | 30.9  |
| % peptide cross-links| 56.5   | 50.9  | 55.1  | 58.3  | 57.8  | 58.5  | 60.4  | 58.8  | 60.0  |

% Values represent the percentage area of each muropeptide from raw data (found in Table 2) calculated to give a total of 100%. Boldface numbers represent a change in relative abundance of ≥±20% from wild type. Boldface numbers with an asterisk represent ±30% change from wild type. Percentages are shown from values rounded to the nearest 0.1%. Muropeptide profiles were generated in two sets of experiments, Sample Set #1 and Sample Set #2. Muropeptide profiles were compared with the wild-type 81-176 muropeptide profile that was analyzed in the same sample set.

Δpata mutant showed an increase in monomeric tripeptides, tetra-penta dipeptides, and tetra-tetra-tripeptides. For ΔpatB, the monomeric tripeptides and dimeric tetra-penta species increased relative to wild type. Δoap exhibited some differences from wild type that followed the same trend as with Δpata, an increase in tetra-penta dipeptides and tetra-tetra-tripeptides, and some that were unique to Δoap, an increase in dipeptides and a decrease in tripeptides. Analyses of PG muropeptide profiles for ΔpataA- and ΔpatB-complemented strains ΔpataC and ΔpatB showed minimal changes from wild type.
with restored O-acetylation levels for \(\Delta\text{pat}B^C\) but not \(\Delta\text{pat}A^C\). The largest number of changes and greatest degree of change occurred in \(\Delta\text{ape}1\). In \(\Delta\text{ape}1\), total dipeptide species decreased and total tripeptides and pentapeptides increased relative to wild type. The total amount of dimers also appeared to be slightly lower in \(\Delta\text{ape}1\). The majority of the muropeptide changes was restored to near wild-type levels in the \(\Delta\text{ape}1\) complement strain (\(\Delta\text{ape}1^C\)).

Recombinant Ape1 Has In Vitro Acetylase Activity on \(p\)-Nitrophenyl Acetate and O-Acetylated PG—Based on the muropeptide data observed for \(\Delta\text{ape}1\) and phenotypic data shown below, the acetylase activity of Ape1 was confirmed biochemically. Ape1 was expressed with a His\(_6\) tag and minus the signal peptide at either the N or C terminus and purified (Fig. 3A and B). Both recombinant proteins purified well (Fig. 3C), producing 3 ml of 0.9–1.2 mg/ml protein after dialysis from a starting culture of 100 ml. The expected sizes of the recombinant proteins are 45.0 and 44.9 kDa for His\(_6\)-Ape1 and Ape1-His\(_6\), respectively. The specific activity of purified Ape1 was determined using \(p\)-nitrophenylacetate (pNPAc), a common substrate used to test esterase activity (31, 32). Specific activity for Ape1-His\(_6\) ranged between 26.1 and 38.9 \(\mu\)mol/min/mg of protein (Fig. 3D), which was higher than the reported specific activity of \(\sim 10.4 \mu\)mol/min/mg for \(N. gonorrhoeae\) Ape1 measured under similar reaction conditions (32). This demonstrates that the recombinant protein exhibits acetylase activity. His\(_6\)-Ape1 showed similar specific activity with pNPAc (data not shown).

Ape1-His\(_6\) was also assayed for acetylase activity on its native substrate (Fig. 3E), O-acetylated PG. PG isolated from \(\Delta\text{ape}1\) was used as the substrate due to the increased PG O-acetylation levels in this strain. Cleavage of O-acetyl groups was assessed using a commercial acetic acid assay kit (Megazyme) as an end point experiment. At 0 h, the average acetate concentration in the sample was 2.4 \(g/ml\) after incubation of \(\Delta\text{ape}1\) PG for 24 h in the absence of enzyme resulted in an average acetate concentration of 35.9 \(g/ml\). Incubation of \(\Delta\text{ape}1\) PG for 24 h in the absence of enzyme resulted in an average acetate concentration of 35.9 \(g/ml\). Incubation of \(\Delta\text{ape}1\) PG for 24 h in the absence of enzyme resulted in an average acetate concentration of 35.9 \(g/ml\).
Role of C. jejuni Peptidoglycan O-Acetylation

with Apel-His$_6$. Because of the insoluble nature of PG, data from this assay cannot be expressed in the classical definitions of enzyme kinetics using the native substrate.

Microscopy and CellTool Analyses of C. jejuni Δape1 Population Morphology Reveal Shape Pleomorphism—Because a number of changes were observed in the muropeptide profiles of the OAP mutants, it was hypothesized that these changes may result in changes in cell shape. The morphology was examined by DICM after 4 and 7 h of growth initiated at an A$_{600}$ of 0.05 to obtain early- and mid-exponential phase cultures (Fig. 4). Whereas wild type exhibited the classical C. jejuni helical shape (Fig. 4A), Δape1 exhibited primarily “comma-shaped” and differentially curved cells (Fig. 4B). Wild-type helical morphology was restored upon complementation (Fig. 4C). A distinct change in morphology was not observed for the other OAP mutant populations (Fig. 4, D–F).

The open-source shape analysis program CellTool (53) was used to quantify the changes in shape in the OAP mutants (Fig. 5). The program contains a set of tools used to extract shapes from binary images that can then be used to assess and compare the population morphology using a variety of metrics. Extracted shapes from the wild-type population were aligned to one another, and principal component analysis (PCA) was performed to generate a baseline model for variation within the wild-type population. At mid-exponential phase, three shape modes (principal components that define specific changes in cell shape in the population) described 95% of the morphological variation in the C. jejuni wild-type population. Each shape mode represented an observable metric (Fig. 5, A and B). Shape mode 1 corresponded to variation in cell length, explaining >90% of the variance (as expected because a population likely exhibits asynchronous growth and division). Shape mode 2 explained 1.9% of the variance and appeared to have some relation to the curvature and wavelength of the cell. A third shape mode explained 1.7% of the variance and described differences in cell width. Contours of mutants were aligned to the wild-type shape model as a reference and Kolmogorov-Smirnov (KS) statistical tests were used to compare sample probability distributions. Based on the large population of bacterial cells assessed and conditions required for KS analysis, a p value of 0.00001 was used as a cutoff for significance (Fig. 5B) (54).

No strains were significantly different from the wild type in shape mode 1 (cell length) or from each other, with the graphical output also showing that the population distributions overlay very closely. In shape mode 2 (cell curvature), some differences in population distribution between wild-type and Δape1, ΔpatA, ΔpatB, and Δoap were significant by the KS cutoff utilized. However, the graphical output showed that these strains were similar to wild type, whereas Δape1 exhibited a dramatic shift in the population distribution maximum (~2.2 standard deviations from the wild-type mean). Shape mode 3 (cell width) was significantly different in all strains compared with wild type (with the exception of Δape1C), and each exhibited a shift of approximately 1 S.D. in the population maximum toward a reduced width compared with the wild-type mean as reflected in the graphical output. A 2D scatterplot of measurements of each individual contour of wild type, Δape1, and Δape1C populations for shape modes 2 and 3 (Fig. 5C) likewise shows that there was a clear difference in shape for the Δape1 population compared with wild type and Δape1C. Early-exponential phase bacteria exhibited similar population shifts as for mid-exponential phase bacteria (with the exception of shape mode 3, as cell width was not captured as a major contributor to the variance in shape for wild type at this time point). The most notable shift at early-exponential phase was observed for cell curvature (shape mode 2) in Δape1 (data not shown).

Phenotypic Analyses Reveal the Importance of O-Acetylpolypeptidoglycan Esterase Activity on Various Aspects of C. jejuni Physiology—The OAP mutants were assessed for different phenotypes serving as indicators of transmission and/or colonization efficiency: motility in soft agar, biofilm formation, hydrophobicity, and sensitivity to a variety of inhibitory compounds. Motility is a major colonization determinant for C. jejuni (55). Although all strains exhibited defective halo formation compared with wild type in soft agar plates after point inoculation (Fig. 6A), the halo diameter of Δape1 was 70% of wild type, whereas ΔpatB, ΔpatA, and Δoap were only modestly defective at 90, 90, and 87% of wild type. Complementation of Δape1 restored the halo formation of the mutant to 90% of wild type and was significantly different from that of Δape1. In addition, Δape1 formed aberrant halos on soft agar with rough perimeters as opposed to the circular halos formed by wild type. This halo phenotype was absent in the other mutants tested and was rescued by complementation.

The ability to form biofilms is important in C. jejuni persistence and transmission. C. jejuni has been shown to survive up to 28 days in a biofilm state and is a general stress response (56). The ability of our OAP mutants to form biofilms was assessed in borosilicate test tubes by crystal violet staining of standing cultures (57). The Δape1 mutant exhibited a hyperbiofilm phenotype, producing 5.5-fold more biofilm than wild type (Fig. 6B). Δape1 also developed flocs of bacteria suspended in the broth (58), which were not observed for wild type nor included in the crystal violet quantification of surface-adhered biofilm (data not shown). Complementation of Δape1 restored biofilm formation to wild-type levels (Fig. 6B). Biofilms formed
Role of C. jejuni Peptidoglycan O-Acetylation

Alignment of C. jejuni wild-type 81-176 cell contours and resultant PCA shape model

A) Alignment of C. jejuni wild-type 81-176 cell contours and resultant PCA shape model

B) Smooth histograms of C. jejuni wild-type 81-176 and OAP mutant populations for each PCA model shape mode and p-values of statistical comparisons

C) Scatter plot of C. jejuni wild-type 81-176, Δape1, and Δape1C

FIGURE 5. CellTool analysis of wild-type strain 81-176, Δape1, Δape1C, ΔpatB, ΔpatA, and Δoap population morphology. Differential interference contrast images were taken of strains grown for 7 h in MH-TV broth at a starting A600 of 0.05 (to mid-exponential phase). Images were converted to binary format (white cells on a black background), and lumps and artifacts were manually removed before processing with CellTool “extract contours function” to generate contours representing each cell (53). A contour extraction, alignment, and generation of the PCA shape model for C. jejuni wild-type strain 81-176. CellTool “align contours” function was used to align the contours of the wild-type population to one another. PCA was performed to generate a wild-type shape model that explains 95% variation in the population in principal components called “shape modes.” Shape modes 1, 2, and 3 represent variation in length, curvature/wavelength, and width, respectively. The extracted contours of the mutant populations were then aligned to the wild-type shape model, and a measurement representing the normalized standard deviation from the wild-type mean in each shape mode was generated and depicted graphically. KS tests were performed for each shape mode between each population and are summarized below the plots. C. jejuni wild-type, Δape1, and Δape1C were plotted with shape mode 2 along the x axis and shape mode 3 along the y axis to create a scatterplot showing the variation in the different populations.

by ΔpatB and ΔpatA were indistinguishable from wild type, but Δoap produced ~2.5-fold more biofilm than wild type. Characterization of Δape1 biofilms by microscopy was unsuccessful as Δape1 formed biofilms poorly on coverslips unlike wild type. This indicates altered cell surface properties in Δape1. Cell surface hydrophobicity was assessed with hexadecane partitioning (Fig. 6C) (59). The percent hydrophobicity of Δape1 was significantly higher (2.0-fold) than wild type and was restored to wild-type levels upon complementation.

The sensitivity of the OAP mutants to detergents, salts, and antimicrobial compounds was tested by determining the minimum inhibitory concentration that reduces growth by 50% relative to a control as measured by A600 (MIC50) (Table 4). Only Δape1 exhibited an increased susceptibility to any of the compounds tested as follows: the amphipathic bile salt sodium deoxycholate (DOC) and MgCl2. For Δape1, an MIC50 range for DOC of 0.16 to 0.31 mg/ml was observed, whereas the MIC50 for wild type was greater than the highest concentration of DOC tested (> 10 mg/ml). Δape1 exhibited a 4–8-fold reduction in MIC50 for MgCl2 compared with wild type. Complementation of Δape1 restored wild type sensitivity profiles.
Role of C. jejuni Peptidoglycan O-Acetylation

A) Motility assays of OAP mutants in soft agar

B) Crystal violet staining of OAP mutant biofilms

C) Cell surface hydrophobicity assessed by hexadecane partitioning

FIGURE 6. Motility in soft agar, biofilm formation, and cell surface hydrophobicity of OAP mutants and wild-type strain 81-176. A, Δape1 exhibits a 30% decrease in halo diameter and abnormal halo formation (rough edges). Motility in soft agar was assessed by measuring the halo diameter after 24 h of strains point-inoculated in 0.4% semi-solid agar. Representative images of halos are shown below each graph. Results shown are representative of one of three independent experiments with 6 replicates. Each strain was compared with wild-type using a paired Student’s t test, with **, ***, and **** indicating p < 0.01, p < 0.001, and p < 0.0001. B, Δape1 and Δoap exhibit 5.5- and 2.5-fold enhanced biofilm formation, respectively, at 24 h. Biofilm formation was assessed after 24 h by crystal violet staining of standing cultures in borosilicate tubes and spectrophotometric quantification of dissolved crystal violet at 570 nm. Results shown for the mutants (left) are representative of one of three independent experiments carried out in triplicate. The results for Δape1C (right) are representative of one of two experiments performed in triplicate. ns, not significant. C, Δape1 exhibited a 2.0-fold increase hydrophobicity relative to wild type, as assessed by hexadecane partitioning. Results are representative of one of three independent experiments performed in triplicate. For biofilm and hydrophobicity, strains were compared using an unpaired Student’s t test, with *, **, ***, and **** indicating p < 0.05, p < 0.01, p < 0.001, and p < 0.0001. Error bars represent standard deviation.

TABLE 4

MIC<sub>50</sub> of C. jejuni OAP mutants determined by broth dilution

Measurements indicated with a “-” have not been tested. Measurements in boldface were consistently ≥4-fold different from wild type over three experiments. MIC<sub>50</sub>, minimum inhibitory concentration to reduce growth by 50% as assessed by optical density.

| Compound          | 81-176 | Δape1  | ΔpatB | ΔpatA | Δoap | Δape1<sup>C</sup> |
|-------------------|--------|--------|-------|-------|------|-------------------|
| Detergents        |        |        |       |       |      |                   |
| Deoxycholate (mg/ml) | >10    | 0.16–0.31 | 5–10 | 1.3–10 | 1.3–10 | 1.3–10           |
| SDS (mg/ml)       | 10–12.5 | 2.5–6.25 | 10–12.5 | 2.5–12.5 | 5–6.25 | 12.5             |
| Triton (% v/v)    | 0.05   | 0.02–0.005 | 0.02–0.05 | 0.02–0.05 | 0.05 | 0.05              |
| Antimicrobials    |        |        |       |       |      |                   |
| Ampicillin (µg/ml) | 2.4–4.9 | 1.2–4.9 | 4.9   | 4.9   | 2.4–4.9 | 2.4              |
| Lysozyme (mg/ml)  | >5     | >5     | >5    | >5    | >5    | >5               |
| Polymyxin B (µg/ml) | 20    | 10     | 10    | 10    | 10    | 10–20            |
| Protamine (µg/ml) | 31.3   | 15.6–31.3 | 15.6 | 15.6 | 31.3–62.5 |                   |
| Chelating agent   |        |        |       |       |      |                   |
| EDTA (µM)         | 78–156 | 1.2–156 | 156   | 156   | 156   | 156              |
| Salts             |        |        |       |       |      |                   |
| NaCl (mM)         | 62.5–250 | 31.3–62.5 | 62.5–125 | 125   | 62.5–125 | 62.5–125         |
| MgCl<sub>2</sub> (mM) | 62.5–125 | 15.6  | 62.5–125 | 125   | 62.5–125 | 62.5–125         |
| CaCl<sub>2</sub> (mM) | 125   | 125–250 | 125–250 | 125–500 | 500   | 125              |
| KCl (mM)          | 62.5   | 31.3–62.5 | 62.5  | 31.3–62.5 | 125   | 31.3–62.5        |

Ape1 Is Required for C. jejuni Bacteria-Host Interactions—

The contribution of OAP to C. jejuni host interactions was examined by determining recovery of the mutants after chick colonization and host cell infections, as well as the ability to elicit IL-8 secretion in vitro in human epithelial infections.

Chickens are an avian reservoir for C. jejuni and a common source of human infection. The Δape1 mutant exhibited a significant 4.4-log decrease in colonization (Fig. 7A), whereas ΔpatB, ΔpatA, and Δoap mutants were not defective for chick colonization. The defects in long term survival in broth for the OAP-deficient mutants were modest compared with Δape1, so the defect in chick colonization could be related to the altered long term survival properties of Δape1.

The ability of a C. jejuni strain to invade and survive in non-phagocytic epithelial cell lines has been shown to correlate with virulence (11, 60, 61). The ability of the C. jejuni OAP mutants to
Role of \emph{C. jejuni} Peptidoglycan O-Acetylation

![Graph showing the effect of OAP on bacterial interactions]

**FIGURE 7. Effect of OAP levels on \emph{C. jejuni} host-bacteria interactions.** A, \emph{Δape1} shows reduced chick colonization compared with wild-type strain 81-176, whereas \emph{ΔpatB}, \emph{ΔpatA}, and \emph{Δoap} mutants display wild-type colonization. Each point represents the recovery of \emph{C. jejuni} strains in log CFU/g of cecal contents from individual day-old chicks 6 days post-colonization with 1 × 10^6 CFU/ml of the indicated strain. The geometric mean is denoted by a black bar. Error bars represent 95% confidence intervals. Adherence, invasion, and intracellular survival of \emph{C. jejuni} in INT407 epithelial cells were assessed by a Gm protection assay and OAP mutant strains. \emph{Δape1} (B) shows a reduced ability to adhere to, invade, and survive in INT407 epithelial cells that were restored upon complementation (C). \emph{ΔpatB} (D), \emph{ΔpatA} (E), and \emph{Δoap} (F) exhibit near wild-type adherence, invasion, and intracellular survival properties. INT407 cells were infected with \emph{C. jejuni} at a multiplicity of infection of ~80. Adherence and invasion were quantified at 3 h post-infection. At this point, the media in the remaining wells were replaced with MEM containing gentamicin (150 µg/ml) and incubated for 2 h, after which the amount of bacterial cells that had invaded the epithelial cells was measured (5-h invasion time point). The Gm in the remaining wells was washed off, and the cells were incubated with fresh MEM containing 3% FBS and a low dose of Gm (10 µg/ml) for an additional 3 h (8-h intracellular survival time point). CFU/ml was determined for each well by lysing the cells with water and plating the dilutions onto MH-TV plates. Results for \emph{B} are representative of two independent experiments performed with three biological replicates.

Discussion

PG plays roles in multiple facets of bacterial physiology. PG modifications have been shown to influence pathogenic properties in several bacterial species (15, 20, 36). Here, the OAP genes in \emph{C. jejuni} were shown to contribute to PG O-acetylation, consistent with their predicted functions. These genes were also important for several key physiological and pathogenic properties. This was most notable for \emph{ape1}, which was involved in PG de-O-acetylation and the only OAP gene significantly required for every phenotype examined.

Deletion of \emph{patA} or \emph{patB}, which act to O-acetylate MurNAc, was non-lethal as found in several other bacterial species (33, 37, 44). This suggests that O-acetyl groups added by PatA/B play a non-essential role for growth of \emph{C. jejuni} in the laboratory. Unlike in \emph{N. meningitidis} where OAP is exclusively mediated by \emph{patA/B} (33), the O-acetylation levels were not reduced to 0% in \emph{C. jejuni}, indicating the presence of alternative PG O-acetylation machinery or compensation by alternative mechanisms, as was observed with \emph{E. coli} WecH that acted as an acetate transporter (62). Expression of \emph{N. gonorrhoeae} PatB in \emph{E. coli} increased OAP levels from ~0.05 to 1%, which was detrimental to the cells (62). The effect of low levels of OAP on \emph{E. coli} biology provides support that the residual O-acetylation...
Role of C. jejuni Peptidoglycan O-Acetylation

in C. jejuni may be sufficient to mask mutant phenotypes in ΔpatA/B/opa that would otherwise be observed if PG O-acetylation were completely absent. There are conflicting results for the essentiality of ape1 in N. gonorrhoeae (43, 44). In C. jejuni, ape1 was not essential. Deletion of ape1 resulted in increased O-acetylation levels almost triple that of wild type, supporting the role of Ape1 in C. jejuni PG de-O-acetylation. Ape1 acety-esterase activity was also confirmed in vitro using the artificial substrate pNPAC as well as its natural substrate, O-acetylated PG from Δape1.

It should be noted that the OAP levels of wild-type C. jejuni 81-176 described here were lower than those reported in a previous study for ATCC 700819 and NCTC 11168 (42). However, the strains and growth conditions used differed between the studies. A direct comparison of how these and other potential factors might affect C. jejuni PG O-acetylation has not yet been assessed but will be the topic of future work.

As with the OAP analyses described above, the muropeptide profiles also showed differences in PG O-acetylation levels for the C. jejuni OAP mutants. Although these data are not truly quantitative for O-acetylation, they offer additional qualitative support for the role of these OAP genes in PG O-acetylation. N. meningitidis Δape1 showed an increase in only O-acetylated muropeptides with a tri-peptide stem in comparison with wild type (33), although the muropeptide analysis of C. jejuni Δape1 suggests that Ape1 in C. jejuni may be regulated differently, as this specificity was not observed. Previous observations with N. gonorrhoeae PatB O-acetyltransferase using in vitro assays showed specificity of PatB toward O-acetylation of tetrapeptides (30). A decrease in O-acetylated tetrapeptide species was observed for the C. jejuni ΔpatA and ΔpatB mutants (Table 2); however, as this could have been due to hydrolysis during the preparation procedure, further biochemical analysis will be required.

As expected, Δape1 also exhibited a 42.1% decrease in relative anhMP levels (presumably due to impaired LT activity) and a greater average chain length. Although chain length was not directly measured, these data support previous findings that Ape1 regulates PG chain length as in N. meningitidis (33). The anhMP levels changed only marginally in ΔpatA, ΔpatB, and Δopa in vitro assays showed specificity of PatB toward O-acetylation of tetrapeptides (30). A decrease in O-acetylated tetrapeptide species was observed for the C. jejuni ΔpatA and ΔpatB mutants (Table 2); however, as this could have been due to hydrolysis during the preparation procedure, further biochemical analysis will be required.

Differences in the muropeptide composition could be possible if O-acetyl groups influence substrate recognition by PG remodeling enzymes. Care must be taken in interpreting how differences in relative abundance actually affect overall PG composition. For instance, small changes in muropeptides of low abundance can result in changes ±20% (i.e. total penta-Gly-5 species, which were 0.8% in wild type and 1.3% for Δape1; Table 3). Conversely, larger changes in muropeptides of high abundance can produce changes <20% yet may still be considered significant. For example, dimeric species constituted 47.7% of the muropeptides in wild type and 40.7% in Δape1; this degree of change may be meaningful, as it affects 7% of the total muropeptides, is unique compared with other mutants tested, and would be considered significant using the 10% cutoff described for H. pylori (27). Regardless, it is clear that the absence of ape1 affects the PG muropeptide profile more than the absence of patA/B (Table 3 and Fig. 2). These changes could be a result of increased O-acetylation affecting substrate recognition by PG remodeling enzymes or missing protein-protein interactions in the absence of Ape1 and will require more extensive analysis in later studies.

N. meningitidis Ape1 showed preference for O-acetylated tripeptide substrates in vivo (33), as mentioned above. The crystal structure for N. meningitidis Ape1 has recently been solved (46), confirming its classification as a member of the Ser-Gly-Asn-His (SGNH) hydrolase superfamily based on active site catalytic residues (32). These residues are also conserved in C. jejuni Ape1. The putative PG binding domain in the N-terminal lobe of N. meningitidis Ape1 and its interaction with PG have yet to be described (46). As N. meningitidis Ape1 was active against various O-acetylated muropeptides in vitro, specificity may be due to regulation of activity through unknown interaction partners. The putative PG binding domain at the N terminus may confer substrate specificity (33). There is only 26/42% amino acid sequence identity/similarity between the N-terminal domains of N. meningitidis Ape1 and C. jejuni Ape1, so the two enzymes may possess different regulatory regions. Another possibility is that C. jejuni lacks the Ape1 interaction partners present in N. meningitidis conferring substrate specificity.

One of C. jejuni’s defining characteristics is its helical shape, a trait defined by the cytoskeleton-like components that coordinate the PG biosynthetic machinery (66). The muropeptide composition was altered in C. jejuni/H. pylori periplasmic PG hydrolase mutants, i.e. Δppg1/Δcsd4 and Δppg2/Δcsd6, and exhibited a straight rod versus helical morphology (18, 19, 28, 67). Deletion of C. jejuni Δape1 also resulted in altered muropeptide composition and shape, but the change in shape was not as dramatic as in the abovementioned straight-rod mutants. Thus, CellTool was employed for shape quantification. This analysis showed that Δape1 was significantly different from the wild-type population in curvature in that it had both an average shape with a larger wavelength compared with wild type and a greater variance of curvature within the population. Ape1 was shown to affect cell size in N. meningitidis (33). In this study, there was a significant increase in total area of Δape1 cells when compared with wild type at early-exponential phase but not at mid-exponential phase (data not shown). One explanation could be that Ape1 activity varies at different growth stages in C. jejuni.

Multiprotein flagellar complexes span the PG layer with some proteins of the complex proposed to directly interact with PG. These proteins include FlG1, which makes up the P-ring of the periplasmic rod-structure in the hook-basal body (55, 68, 69), and MotB in H. pylori that makes up part of the flagellar stator responsible for generating torque (70). In Salmonella enterica, the switch protein FlIG of the C-ring, which acts as the rotary component of the flagella, responds to chemotactic sig-
acetylation and destabilization of the outer membrane by adding EDTA (at MIC_{so} and concentrations down to 4-fold less than MIC_{so}) or lactoferrin (at physiological concentration of 3 mg/ml, as described previously (37)) to the lysozyme incubations also failed to result in differential lysozyme sensitivity (data not shown). Lysozyme turbidometric assays were also unsuccessful due to the low yield of PG, resulting in an initial absorbance reading too low to accurately detect a response (data not shown).

Chick colonization by Δape1 was significantly impaired compared with wild type and ΔpatA, ΔpatB, and Δoap colonized to wild-type levels (Fig. 6A). Motility and chemotaxis are important for colonization (79); thus, this could be a potential explanation for the Δape1 chick data. Alternatively, and/or additionally, the altered morphology and PG structure (18, 19), increased DOC susceptibility, differential long term survival properties, and other as-yet unknown factors could also contribute to the Δape1 colonization defect.

Of the OAP mutants, only Δape1 was impaired in adherence, invasion, and intracellular survival in INT407 epithelial cells. Whether these observations represent defects at each time point, defects in adherence that in turn affect recovery at later time points, or if Δape1 is very rapidly killed upon invasion (as the 3-h “adherence” time point will also reflect invaded bacteria) will require further experimentation. Infection of human INT407 epithelial cells by Δape1 also led to a decrease in IL-8 secretion. This may correlate with its reduced invasion properties.

Somewhat surprisingly, the reduction in PG O-acetylation had no significant effects on colonization, host cell interactions, or any other phenotype examined except for marginal decreases in halo formation, suggesting that under these conditions OAP by PatA and PatB offers no fitness advantage in host survival, which is perplexing and leaves the role of PG O-acetylation in C. jejuni yet to be determined. In contrast, the increase in O-acetylated PG in Δape1 was detrimental to C. jejuni in multiple aspects important for pathogenesis. Future studies will focus on finding a direct link between PG O-acetylation and the observed changes in physiology, identifying other potential mechanisms of PG O-acetylation and de-O-acetylation, and revealing the underlying cause(s) of the impaired host interactions for Δape1.

**Experimental Procedures**

**Strains and Growth Conditions**—A list of bacterial strains and plasmids used in this study can be found in Table 5. Construction of mutant and complemented strains is described in supplemental text S1 using primers listed in supplemental Table S1. C. jejuni strains, unless otherwise stated, were grown in Mueller-Hinton (MH; Oxoid) broth or agar (1.7% w/v) supplemented with vancomycin (10 μg/ml) and trimethoprim (5 μg/ml) and when appropriate kanamycin (Km; 50 μg/ml) and chloramphenicol (Cm; 25 μg/ml). Standard laboratory conditions for C. jejuni growth were 38°C under microaerophilic conditions (12% CO_{2}, 6% O_{2}, in N_{2}) in a Sanyo tri-gas incubator for MH agar or for standing MH broth cultures. For shaking MH broth cultures (hereafter referred to as broth cultures), C. jejuni were cultured in airtight jars using the Oxoid CampyGen Atmosphere generation system with shaking at 200 rpm. Experiments were performed using cultures initiated at A_{600} 0.002 and grown in shaking broth for 16–18 h to reach exponential phase. For plasmid construction and protein purification...
Role of C. jejuni Peptidoglycan O-Acetylation

### Table 5

| Strain or plasmid | Genotype or description | Source |
|-------------------|-------------------------|--------|
| E. coli strains   |                         |        |
| DH5-α             | F- ϕ80d deR lacZΔM15 endA1 recA1 (81) purC175 rpsL15 m15 t10 hsdR30 dcm L9002 | Invitrogen |
| BL21 (DE3)        | F- ompT hsdS2 (rK-, m-, uA-) gal dcm [DE3] T18 | Novagen |
| Plasmids          |                         |        |
| pGEM-T            | High copy, linearized, T-tailed, Blue/White, ApR | Promega |
| pUC18-K2          | Source of non-polar aphA3 cassette; ApR KmR | 47 |
| pGEM-T:0638       | pGEM-T ligated to 0638 amplified with 0637-2 and 0639-5 (2113 bp); ApR | This study |
| pGEM-0638:aphA-3  | pGEM-T:0638 inverse PCR amplified with 0638-2 and 0638-3 (4098 bp) and ligated to aphA-3 (KpnI, HincII); ApR, KmR | This study |
| pGEM-T:0639       | pGEM-T:0639 amplified with 0639-1 and 0639-2 (2146 bp); ApR | This study |
| pGEM-0640:aphA-3  | pGEM-T:0640 inverse PCR amplified with 0640-1 and 0640-2 (4168 bp) and ligated to aphA-3 (KpnI, HincII); ApR, KmR | This study |
| pGEM-0640:0638-40:aphA-3 | pGEM-T:0640:aphA-3 amplified with 0640-1 and 0640-2 (4168 bp) and ligated to aphA-3 (KpnI, HincII); ApR, KmR | This study |
| pRRC              | C. jejuni RNA spacer integration vector; CmR | 48 |
| pRRC:0638         | pRRC ligated to 0638 amplified with 0638-C1 (Ndel) and 0638-C2 (MfeI) (1347 bp); CmR | This study |
| pRRC:0639         | pRRC ligated to 0639 amplified with 0639-C1 (Ndel) and 0639-C2 (MfeI) (1276 bp); CmR | This study |
| pRRC:0640         | pRRC ligated to 0640 amplified with 0640-C1 (Ndel) and 0640-C2 (MfeI) (1616 bp); CmR | This study |
| pET28a(+);0636    | Commercial vector for expression of recombinant His6-tagged protein | Novagen |
| pHi6-A::0638      | pET28a (+) ligated to 0638 amplified with 0638-ecF (NcoI) and 0638-ecR (EcoRI) (1121 bp) for expression of C-terminal His6-tagged 0638 | This study |
| pHi6-A::0638-1166 | pET28a (+) ligated to 0638 amplified with 0638-ecF (NcoI) and 0638-ecR (EcoRI) (1116 bp) for expression of N-terminal His6-tagged 0638 | This study |

E. coli (DH5-α or BL21) strains were grown at 37°C in Luria-Bertani (LB; Sigma) broth or LB agar (7.5% w/v) supplemented with ampicillin (100 μg/ml), Km (25 μg/ml) or Cm (15 μg/ml) as required.

PG Isolation and Assessment of O-Acetylation Levels—PG isolation for O-acetylation analysis was performed as described previously with minor modifications (32, 51). Each strain was grown on ~60 MH-T agar plates (supplemented with Km or Cm as necessary) for ~18–20 h. The cells were harvested from the plate with 1 ml of ice-cold MH broth per plate and added to a conical tube. Strains were assessed by DICM to examine for contamination and the presence of coccolid cells, ensuring that the cultures had not entered stationary phase. Cells were lysed by DICM for contamination and coccoid cells to ensure that cultures had not entered stationary phase. Cultures had not grown into stationary phase. Cells were lysed using the boiling SDS technique as described previously (19). PG was further purified from the cell lysate and digested with the muramidase cellosol (kindly provided by Hoechst, Frankfurt, Germany). The muropeptides were reduced with sodium borohydride, and subsequently separated by HPLC, all as described previously (81). Muropeptide structures were assigned based on (i) comparison with retention times of known muropeptides from C. jejuni and (ii) by mass spectrometry (Fig. 2 (18, 19, 82).

Expression, Purification, and Biochemical Assays of C. jejuni Ape1-His6—Cj81176_0638 (encoding ape1) was PCR-amplified without the predicted 21-amino acid signal peptide (as...
identified by SignalP 4.1 Server (83) and cloned into the pET28a (+) (Novagen) expression vector in-frame with the encoded His6 tag at either the N or C termini of the gene forming pHis6-0638 and p0638-His6, respectively (Fig. 3, A and B). Expression constructs were transformed into E. coli BL21(ADE3), selected for Km, and confirmed by PCR and sequencing. Expression and isolation of recombinant Ape1 are described in the supplemental text S2.

Acetyltransferase activity was determined using pNPAc as a colorimetric substrate, as described previously (45). Assays were performed with 2.5 μg/ml purified Ape1-His6 in 50 mM sodium phosphate buffer, pH 6.5, and 2 mM pNPAc. Reactions were monitored over 5 min, and specific activity was calculated with an experimentally determined molar absorptivity of 3.42 mM⁻¹ cm⁻¹ for p-nitrophenol (room temperature at pH 6.5).

PG-O-acetyltransferase activity was tested using purified PG retaining O-acetyl groups (as described under “PG Isolation and Assessment of O-Acetylation Levels”). Lyophilized PG was resuspended in sodium phosphate buffer, pH 6.5, to a concentration of 5 mg/ml and sonicated on ice with a microtip for 2 min (10 s on/10 s off). 500 μL of PG suspension was aliquoted into Eppendorf tubes to which, Ape1-His6 was added to a concentration of 10 μg/ml (buffer only for negative control). Samples were incubated at 37 °C in a water bath for 24 h, after which samples were centrifuged (10 000 × g, 10 min, 4 °C) to pellet PG. Acetate content in supernatants were assessed using a commercial acetic acid assay kit (Megazyme International) as directed by the manufacturer.

**Microscopy and CellTool Shape Analysis**—Overnight MH-TV log-phase broth cultures were standardized to A₆₀₀ 0.05 and incubated for 4 or 7 h at 38 °C to generate early-exponential phase and mid-exponential phase bacterial cultures, respectively. The samples were processed for DICM. Live cells were imaged on agarose slabs on a Nikon Eclipse TE2000-U microscope equipped with a Hamamatsu C4742-95 digital camera.

For CellTool analysis (53), DICM images from multiple fields (yielding ≥400 cells per strain) were taken for each sample and processed by thresholding to generate binary images. Artifacts and cells that were clumped or ill-represented based on lighting were manually removed. The contours of the wild-type population were aligned to generate an average shape, and PCA was performed to generate a “shape model” based on principal components called “shape modes” that, together, describe at least 95% of the variation in the wild-type population. Contours of other strains were then aligned to the wild-type PCA shape model as a reference. Kolmogorov-Smirnov tests were used on each shape mode to determine whether there was a statistically significant difference in population distribution between the strains based on this wild-type shape model (53).

**Phenotypic Characterization of OAP Mutants: Motility, Biofilm, Minimum Inhibitory/Bactericidal Concentrations and Cell Surface Hydrophobicity**—Motility and biofilm formation assays were performed on log-phase bacterial broth cultures as described previously (19).

MIC₅₀ was determined in a 96-well plate as standing culture as described previously (84). Inocula of log-phase overnight cultures (100 μl) standardized to A₆₀₀ 0.0002 (10⁶ CFU/ml) in MH-TV and 11 μl of 10× concentrated test compound (in 2× serial dilutions) were added to each well. A₆₀₀ was measured for each well using the Varioskan Flash Multimode Plate Reader (Thermo Scientific), and MIC₅₀ was recorded as the lowest concentration of compound that reduced growth by 50% (by turbidity) relative to a positive control after 24 h.

Cell surface hydrophobicity was assessed using exponential phase bacterial broth cultures as described previously (85) with the following adjustments. Cultures were harvested at 8 000 × g for 10 min and washed three times with PBS. Cells were resuspended to an A₆₀₀ ~0.5 in PBS, and the absorbance was recorded. Hexadecane was added to the standardized cultures in a ratio of 1:4 hexadecane/culture by volume, vortexed for 5 min, and incubated at 38 °C for 30 min. The aqueous layer was isolated, aerated by bubbling N₂ gas through the aqueous layer for 30 s, and left open to the air for 10 min to ensure removal of all traces of hexadecane, and the A₆₀₀ was measured. Cell surface hydrophobicity was expressed as follows, where A₆₀₀ refers to the optical densities before and after extraction, respectively.

% Hydrophobicity = \( \frac{A_{600} - A_{600\text{f}}}{A_{600}} \times 100 \) (Eq. 1)

**Chick Colonization**—Chick colonization was assessed under protocol 10462 approved by the University of Michigan Committee on Care and Use of Animals, as described previously (19, 79).

**Gentamicin Protection Assay for Host-Cell Infection**—Gm protection assays were performed essentially as described previously (86). INT407 human epithelial cells were seeded into 24-well tissue culture plates at ~1.25 × 10⁵ cells in minimum essential medium (MEM) supplemented with 10% (v/v) FBS and 1× penicillin/streptomycin (Gibco, Life Technologies, Inc.) 24 h prior to infection. Infections were initiated by adding log-phase bacterial cultures standardized to A₆₀₀ 0.002 in MEM (1 × 10⁷ CFU/ml) and added to INT407 cells previously washed twice with 1 ml of MEM to give a multiplicity of infection of ~80. Adherence/invasion after 3 h of infection, invasion following a 2-h Gm treatment (150 μg/ml) to kill extracellular bacteria, and intracellular survival following removal of the high Gm concentration and incubation of cells in fresh medium with 10 μg/ml Gm and 3% FBS for an additional 3 h were assessed as described (86).

**Interleukin-8 Quantification**—The concentration of IL-8 secreted by INT407 cells either left uninfected or infected with C. jejuni wild-type strain 81-176, Δape1, or Δape1C for 24 h was assayed using the human IL-8 ELISA kit (Thermo Fisher Scientific) as described previously (18).

**Author Contributions**—Initial conceptualization of the project details was by E. F. and E. C. G. R. H. conducted most of the experiments in the study and prepared the manuscript with E. C. G. and E. F. D. S conducted the O-acetylation assays on purified PG from OAP mutants. J. B. conducted experiments on the muropeptide profiles of the OAP mutants. M. E. T. and J. G. J. conducted the chick colonization assays with OAP mutants. Oversight of the project was provided by E. C. G., A. J. C., W. V., and V. J. D. All authors reviewed and approved the final version of the manuscript.
Role of C. jejuni Peptidoglycan O-Acetylation

Acknowledgment—We thank Jenny Vermeulen for technical assistance in PG sample preparations.

References

1. Yuki, N., and Hartung, H. P. (2012) Guillain-Barre syndrome. N. Engl. J. Med. 366, 2294–2304
2. Jacobs, B. C., Rothbarth, P. H., van der Meché, F. G., Herbrink, P., Schmitz, P. L., de Klerk, M. A., and van Doorn, P. A. (1998) The spectrum of ante-cedent infections in Guillain-Barre syndrome: a case-control study. Neurology 51, 1110–1115
3. Man, S. M. (2011) The clinical importance of emerging Campylobacter species. Nat. Rev. Gastroenterol. Hepatol. 8, 669–685
4. Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeira, P. (2011) Campylobacter spp. as a foodborne pathogen: a review. Front. Microbiol. 2, 200
5. Luangtongkum, T., Jeon, B., Han, J., Plummer, P., Logue, C. M., and Logue, C. M. (2007) Characterization of peptidoglycan acetyltransferases in Campylobacter jejuni. FEMS Microbiol. Lett. 270, 445–451
6. Dasti, J. I., Tareen, A. M., Lugert, R., Zautner, A. E., and Gross, U. (2010) Characterization of peptidoglycan acetyltransferases in Campylobacter jejuni. Int. J. Med. Microbiol. 300, 205–211
7. Gundogdu, O., Bentley, S. D., Holden, M. T., Parkhill, J., Dorrell, N., and Wren, B. W. (2007) Re-annotation and re-analysis of the Campylobacter jejuni NCTC11168 genome sequence. BMC Genomics 8, 162
8. Parkhill, J., Wren, B. W., Mungall, K., Kettle, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pillai, M. J., Penn, C. W., Rutherford, K., Whitehead, S., and Whitehead, S. (2000) The Campylobacter jejuni genome sequence: implications for mechanism of pathogenesis. J. Bacteriol. 182, 2141–2151
9. Louwen, P. P., van Belkum, A., Wagenaar, J. A., Doorduyn, Y., Achtberg, R., and Endtz, H. P. (2006) Lack of association between the presence of the pVir plasmid and bloody diarrhoea in Campylobacter jejuni enteritis. J. Clin. Med. 4, 1867–1868
10. Tracz, D. M., Keelan, M., Ahmed-Bentley, J., Gibreel, A. J., and Clarke, A. J. (2013) Identification of the first known peptidoglycan modifying enzyme Pgp1 in Campylobacter jejuni. Cell. Mol. Biol. 59, 8–27
11. Szymanski, C. M., and Gaynor, E. C. (2012) How a sugary bug gets into eukaryotic cells and virulence. Infect. Immun. 80, 4458–4459
12. Eid, M. A., Elsawy, M. H., and Gótz, F. (2006) The presence of Campylobacter jejuni is associated with increased urinary tract infection. J. Antimicrob. Chemother. 57, 1501–1506
13. Moynihan, P. J., and Clarke, A. J. (2014) Substrate specificity of the Campylobacter jejuni O-acetylation pathways. J. Bacteriol. 196, 7008–7019
14. Moynihan, P. J., and Clarke, A. J. (2014) Substrate specificity and kinetic characterization of Campylobacter jejuni O-acetyltransferases. J. Bacteriol. 196, 7010–7019
15. Pfeffer, J. M., and Clarke, A. J. (2012) Identification of the first known peptidoglycan modifying enzyme Pgp1 in Campylobacter jejuni. Cell. Mol. Biol. 58, 8–27
16. Young, K. D. (2006) The selective value of bacterial shape. Microbiol. Mol. Biol. Rev. 70, 660–703
17. Frirdich, E., Biboy, J., Adams, C., Lee, J., Ellermeier, J., Gielda, L. D., Drits, V. I., Girardin, S. E., Vollmer, W., and Gaynor, E. C. (2014) Peptidoglycan-LD-carboxypeptidase Pgp2 influences Campylobacter jejuni helical cell shape and pathogenic properties. Cell. Mol. Biol. 60, 576–582
18. Viala, J., Chaput, C., Bonca, I. G., Cardona, A., Girardin, S. E., Moran, A. P., Athman, R., Ménet, S., Huerre, M. R., Coyle, A. J., DiStefano, S. P., Sansonetti, P. J., Labigne, A., Bertin, J., Philpott, D. J., and Ferrero, R. L. (2004) Nod1 responds to peptidoglycan delivered by Helicobacter pylori cag pathogenicity island. Nat. Immunol. 5, 1166–1174
19. Vollmer, W., and Tomasz, A. (2002) Peptidoglycan N-acetylglucosamine deacetylase, a putative virulence factor in Streptococcus pneumoniae. Infect. Immun. 70, 7176–7178
20. Boneca, I. G. (2005) The role of peptidoglycan in pathogenesis. Curr. Opin. Microbiol. 8, 46–53
21. Cabanes, D., Dussurget, O., Dehoux, P., and Cossart, P. (2004) Auto, a surface associated autolysin of Listeria monocytogenes required for entry into eukaryotic cells and virulence. Mol. Microbiol. 51, 1601–1614
22. Lenz, L. L., Mohammadi, S., Geissler, A., and Portnoy, D. A. (2003) SecA2-dependent secretion of autolytic enzymes promotes Listeria monocytogenes pathogenesis. Proc. Natl. Acad. Sci. U.S.A. 100, 12432–12437
23. Viala, J., Chaput, C., Bonca, I. G., Cardona, A., Girardin, S. E., Moran, A. P., Athman, R., Ménet, S., Huerre, M. R., Coyle, A. J., DiStefano, S. P., Sansonetti, P. J., Labigne, A., Bertin, J., Philpott, D. J., and Ferrero, R. L. (2004) Nod1 responds to peptidoglycan delivered by Helicobacter pylori cag pathogenicity island. Nat. Immunol. 5, 1166–1174
24. Tracz, D. M., Keelan, M., Ahmed-Bentley, J., Gibreel, A. J., and Clarke, A. J. (2012) Changes in peptidoglycan structure and metabolism during differentiation of Proteus mirabilis into swarmer cells. Curr. J. Microbiol. 58, 1183–1194
25. Yu, P. F., and Cottam, P. J. (2005) Peptidoglycan LD-carboxypeptidase Pgp2 influences Campylobacter jejuni helical cell shape and pathogenic properties. Mol. Microbiol. 58, 8007–8018
Role of C. jejuni Peptidoglycan O-Acetylation

41. Moynihan, P. J., and Clarke, A. J. (2011) O-Acetylated peptidoglycan: controlling the activity of bacterial autolysins and lytic enzymes of innate immune systems. Int. J. Biochem. Cell Biol. 43, 1655–1659

42. Weadge, J. T., Pfeffer, J. M., and Clarke, A. J. (2005) Identification of a new family of enzymes with potential O-acetylpeptidoglycan esterase activity in both Gram-positive and Gram-negative bacteria. BMC Microbiol. 5, 49

43. Weadge, J. T., and Clarke, A. J. (2006) Identification and characterization of O-acetylpeptidoglycan esterase: a novel enzyme discovered in Neisseria gonorrhoeae. Biochemistry 45, 839–851

44. Dillard, J. P., and Hackett, K. T. (2005) Mutations affecting peptidoglycan acetylation in Neisseria gonorrhoeae and Neisseria meningitidis. Infect. Immun. 73, 5697–5705

45. Weadge, J. T., and Clarke, A. J. (2007) Identification and characterization of Neisseria gonorrhoeae O-acetylpolyhexosamine lyase. Biochemistry 46, 4932–4941

46. Williams, A. H., Veyrier, F. J., Bonis, M., Michaud, Y., Raynal, B., Taha, M. K., White, S. W., Haouz, A., and Boneca, I. G. (2014) Visualization of a substrate-induced productive conformation of the catalytic triad of the Neisseria meningitidis peptidoglycan O-acetylerase reveals mechanistic conservation in SGNH esterase family members. Acta Crystallogr. D Biol. Crystallogr. 70, 2631–2639

47. Ménard, R., Sansonetti, P. J., and Parsot, C. (1993) Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of Shigella flexneri entry into epithelial cells. J. Bacteriol. 175, 5899–5906

48. Karlyshev, A. V., and Wren, B. W. (2005) Development and application of an Insertional system for gene delivery and expression in Campylobacter jejuni. Appl. Environ. Microbiol. 71, 4004–4013

49. Clarke, A. J. (1993) Compositional analysis of peptidoglycan by high-performance anion-exchange chromatography. Anal. Biochem. 212, 344–350

50. Clarke, A. J. (1993) Extent of peptidoglycan O-acetylation in the tribe proteae. J. Bacteriol. 175, 4550–4553

51. Dupont, C., and Clarke, A. J. (1991) Dependence of lysozyme-catalysed solubilization of Proteus mirabilis peptidoglycan on the extent of O-acetylation. Eur. J. Biochem. 195, 763–769

52. Scheurwater, E., Reid, C. W., and Clarke, A. J. (2008) Lytic transglycosylases: bacterial space-making autolysins. Int. J. Biochem. Cell Biol. 40, 586–591

53. Pincus, Z., and Theriot, J. A. (2007) Comparison of quantitative methods for cell-shape analysis. J. Microsc. 227, 140–156

54. Lin, M. F., Lucas, H. C., and Shmueli, G. (2013) Too big to fail: large samples and the p-value problem. Inform. Syst. Res. 24, 906–917

55. Lertsetthakarn, P., Otttemann, K. M., and Hendrixson, D. R. (2011) Motility and chemotaxis in Campylobacter and Helicobacter. Annu. Rev. Microbiol. 65, 389–410

56. Buswell, C. M., Herlihy, Y. M., Lawrence, L. M., McGuigan, J. T., Marsh, P. D., Keevil, C. W., and Leach, S. A. (1998) Extended survival and persistence of Campylobacter spp. in water and aquatic biofilms and their detection by immunofluorescence-antibody and -rRNA staining. Appl. Environ. Microbiol. 64, 733–741

57. McLennan, M. K., Ringoir, D. A., Firdide, E., Svensson, S. L., Wells, D. H., Jarrell, H., Szymanski, C. M., and Gaynor, E. C. (2008) Campylobacter jejuni biofilms up-regulated in the absence of the stringent response utilize a calcifactor white-reactive polysaccharide. J. Bacteriol. 190, 1097–1107

58. Joshua, G. W., Guthrie-Irons, C., Karlyshev, A. V., and Wren, B. W. (2006) Biofilm formation in Campylobacter jejuni. Microbiology 152, 387–396

59. Rosenborg, M., Gutnick, D., and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons—a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29–33

60. Everest, P. H., Goossens, H., Butzler, J. P., Lloyd, D., Knutton, S., Ketley, J. M., and Williams, P. H. (1992) Differentiated Caco-2 cells as a model for enteric invasion by Campylobacter jejuni and C. coli. J. Med. Microbiol. 37, 319–325

61. Bacon, D. J., Szymanski, C. M., Burr, D. H., Silver, R. P., Alm, R. A., and Guerry, P. (2001) A phase-variable capsule is involved in virulence of Campylobacter jejuni 81–176. Mol. Microbiol. 40, 769–777
Role of C. jejuni Peptidoglycan O-Acetylation

82. Bui, N. K., Gray, J., Schwarz, H., Schumann, P., Blanot, D., and Vollmer, W. (2009) The peptidoglycan sacculus of Myxococcus xanthus has unusual structural features and is degraded during glycerol-induced myxospore development. J. Bacteriol. 191, 494–505
83. Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786
84. Hancock, R. E. (1999) Hancock Laboratory Methods, Department of Microbiology and Immunology, University of British Columbia, British Columbia, Canada
85. Ben Abdallah, F., Lagha, R., Said, K., Kallel, H., and Gharbi, J. (2014) Detection of cell surface hydrophobicity, biofilm and fimbriae genes in Salmonella isolated from tunisian clinical and poultry meat. Iran J. Public Health 43, 423–431
86. Pryjma, M., Apel, D., Huynh, S., Parker, C. T., and Gaynor, E. C. (2012) FdhTU-modulated formate dehydrogenase expression and electron donor availability enhance recovery of Campylobacter jejuni following host cell infection. J. Bacteriol. 194, 3803–3813
87. Korlath, J. A., Osterholm, M. T., Judy, L. A., Forfang, J. C., and Robinson, R. A. (1985) A point-source outbreak of campylobacteriosis associated with consumption of raw milk. J. Infect. Dis. 152, 592–596
88. Glauner, B., Höltje, J. V., and Schwarz, U. (1988) The composition of the murein of Escherichia coli. J. Biol. Chem. 263, 10088–10095