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Phase variable expression of capsular polysaccharide modifications allows *Campylobacter jejuni* to avoid bacteriophage infection in chickens

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**INTRODUCTION**

Bacteriophages (phages) are the most abundant group of organisms on the planet and are estimated to outnumber their bacterial hosts by 10-fold (Brüssow and Hendrix, 2002). The first step of phage infection is through the specific recognition and attachment of the phage particle to the host cell (Weinbauer, 2004). In general, phages bind to unique host-specific structures, thus allowing them to recognize a suitable host in a mixed bacterial population (Rakhuba et al., 2010). Knowledge of phage receptors is required for implementation of efficient phage therapies against human pathogens – an intervention strategy that has been promoted by the increasing occurrence of antibiotic resistance (Levy and Marshall, 2004; Kutateladze and Adamiak, 2010), yet only a few bacterial phage receptors have been identified in Gram-negative bacteria (for example, Gemski and Stocker, 1967; Stirm and Freund-Molbert, 1971; Gemski et al., 1975; Yu and Mizushima, 1982; Morona et al., 1984).

Capsular polysaccharides (CPS) are highly hydrated polymeric gels that provide a thick (400 nm or more) layer protecting bacteria from hostile environments and host immune defenses, but the CPS can also be a target for phage binding and infection (Stirm and Freund-Molbert, 1971; Roberts, 1996; Comstock and Kasper, 2006). The capsule of the zoonotic foodborne pathogen, *Campylobacter jejuni*, is required for serum resistance, invasion of human epithelial cells, colonization of chickens, and diarrheal disease in ferrets (Bacon et al., 2001; Jones et al., 2004). Furthermore, mutation of several *C. jejuni* CPS genes resulted in phage resistance against a group of phages, but the actual capsular moieties involved

**Keywords:** bacteriophage, *Campylobacter jejuni*, capsular polysaccharide, phase variation, phosphoramidate, methylation

**Abbreviations:** β-D-Rib, ribose; β-D-GalNAc, N-acetylgalactosamine in the fucose configurations; α-D-GlcPα6(NRG), glucuronic acid with 2-amino-2-deoxyglycerol at C-6; MeOPN, O-methyl phosphoramidate; Hep, heptose. The 2-amino-2-deoxyglycerol can also be substituted with an N-ethanolamine modification at C-6.
were not identified (Coward et al., 2006). Only recently a specific component of the C. jejuni capsule was identified as the phase receptor for this species. We showed that phase F336 relies on the O-methyl phosphoramide (MeOPN) modification of the C. jejuni NCTC11168 CPS for adsorption to the host cell surface and infection (Sørensen et al., 2011).

The CPS of C. jejuni is the major component recognized by the Penner serotyping scheme for which 47 different serotypes are known (Guerry et al., 2012). Thus, the CPS are highly diverse in structure and this is reflected by the gene content of the capsular loci in various C. jejuni strains (Karlyshev et al., 2005). In addition, a number of CPS genes possess homopolymeric tracts making them prone to phase variation which allows a rapid on/off switching of the genes and promotes variations in CPS even when the strains have identical gene contents (Linton et al., 2001; St Michael et al., 2002; Karlyshev et al., 2005; McNally et al., 2005; Chen et al., 2008). The CPS of C. jejuni NCTC11168 consists of -2)-β-n-Ribf-(1-5)-3-MeOPN-β-n-GalNAc-(1-4)-α-n-GlcPAn6(NGro)-{1-backbone with a 3,6-di-O-methyl-4-MeOPN-β-glycero-α-L-gulo-Hepp side chain (St Michael et al., 2002; Szymanski et al., 2003; McNally et al., 2005). The CPS biosynthetic locus in strain NCTC11168 includes 28 genes and 6 of these genes (cj1420, cj1421, cj1422, cj1426, cj1429, and cj1437) contain homopolymeric G (polyG) tracts (Parkhill et al., 2000). Even though the function of only three out of the six gene products has been identified, a highly variable presence/absence of methyl, ethanolamine, aminoglycerol, and phosphoramide modifications of the CPS have been observed (St Michael et al., 2002; Szymanski et al., 2003; McNally et al., 2005), suggesting that the remaining genes could encode enzymes involved in synthesis or transfer of these modifications to the CPS glycans. Recently, the cj1421 and cj1422 genes were shown to encode MeOPN transferases attaching MeOPN to the GalβNAc and the Hep residues, respectively (McNally et al., 2007). Similarly, cj1426 was demonstrated to encode the 6-O-Me transferase enzyme (Sternberg et al., 2011).

We previously discovered that resistance toward phase F336 evolved with a high frequency in C. jejuni NCTC11168 due to loss of the MeOPN receptor on the bacterial surface as a result of phase variation in the cj1421 gene encoding the MeOPN-GalβNAc transferase. In addition, deletion of the cj1421 gene in NCTC11168 resulted in phase resistance, proving that MeOPN attached to GalβNAc was a receptor for phase F336 (Sørensen et al., 2011). The aim of the present study was to determine if other phases rely on the MeOPN moiety for infection of C. jejuni and to explore the role of other CPS phase variable modifications for phase infection. Knowledge of phage receptors and development of phage resistance in vivo is essential for implementation of efficient phage therapy against C. jejuni in the chicken gut. We found that not only the MeOPN moiety, but also the phase variable 3-O-Me and 6-O-Me groups in the CPS of C. jejuni NCTC11168 may influence phage sensitivity. Co-infection of chickens with C. jejuni NCTC11168 and phase F336 resulted in bacterial acquisition of phase resistance through a loss of MeOPN attached to GalβNAc of the capsule or the acquisition of the 6-O-Me group. We propose that the constant exposure of C. jejuni to naturally occurring phases in the avian gut selects for different phase variable structures of the CPS resulting in a continuous phage-host co-evolution.

**MATERIALS AND METHODS**

**BACTERIAL STRAINS, MEDIA, AND GROWTH CONDITIONS**

The C. jejuni strains used in this study are listed in Table 1 and were routinely grown on blood agar Base II (Oxoid) supplemented...
Table 2 | Bacteriophages.

<table>
<thead>
<tr>
<th>Categorya</th>
<th>HhaI restriction patternb</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F198</td>
<td>III c</td>
<td>Broiler intestine</td>
<td>Hansen et al. (2007)</td>
</tr>
<tr>
<td>F287</td>
<td>III d</td>
<td>Duck intestine</td>
<td>Hansen et al. (2007)</td>
</tr>
<tr>
<td>F303</td>
<td>III d</td>
<td>Duck abatour</td>
<td>Hansen et al. (2007)</td>
</tr>
<tr>
<td>F326</td>
<td>III d</td>
<td>Duck intestine</td>
<td>Hansen et al. (2007)</td>
</tr>
<tr>
<td>F336</td>
<td>III b</td>
<td>Duck intestine</td>
<td>Hansen et al. (2007)</td>
</tr>
</tbody>
</table>

aCategory is based on genome size (all are 140 kb) as defined in Sails et al. (1998).
bHhaI restriction pattern as determined in Hansen et al. (2007): b, 5 + 2 weak bands; c, 4 bands; d, 3 bands.

with 5% calf blood (BA) or in brain heart infusion (BHI) broth under microaerobic conditions (6% CO₂, 6% O₂, 84% N₂, and 4% H₂) at 37°C. For chicken experiments, bacteria were grown on Karmali agar (Oxoid) supplemented with Campylobacter selective supplement under microaerobic conditions (10% CO₂, 5% O₂, 85% N₂) at 37°C.

**BACTERIOPHAGES**

Bacteriophages used in this study are listed in Table 2. They all belong to the family of Myoviridae and have genome sizes of approximately 140 kb, but show different HhaI restriction patterns. Phages were propagated on *C. jejuni* NCTC12662, except for phage F336 where *C. jejuni* 1447 was used (Hansen et al., 2007). Stock bacteriophages were kept in SM buffer (0.05 M Tris–Cl, pH 7.5 supplemented with 5.8 g NaCl, 2.0 g MgSO₄·7H₂O, and 5 ml gelatin, 2% w/v solution) at 4°C.

**BACTERIOPHAGE PROPAGATION**

Bacteriophage propagation was performed as described by Sørensen et al. (2011) using a plate lysis method developed from Frost et al. (1999), Hansen et al. (2007). The propagating strains were grown overnight on blood agar Base II plates and harvested in BHI supplemented with 1 mM CaCl₂ and 10 mM MgSO₄ (CBHI). Bacterial suspensions were adjusted to an optical density of OD₆₀₀ = 0.35, incubated for 4 h at 37°C under microaerobic conditions, mixed with phages at a multiplicity of infection (MOI) of 0.01 and incubated for 15 min at 37°C to allow bacteriophage adsorption. Subsequently, 0.6 ml of the adsorbed suspensions were mixed with 5 ml of NZCYM overlay agar [NZCYM (Sigma) broth with 0.6% agar] at 45°C and poured onto CBHI 1.2% agar plates. After incubation, plates with lysis were flooded with sterile SM buffer and the phages eluted overnight with gentle shaking at 4°C. Phage suspensions were harvested and filtered through a sterile 0.2 μm membrane filter.

**BACTERIOPHAGE TITRATION**

Bacteriophage titration was performed as previously described (Sørensen et al., 2011). Briefly, bacterial lawns were made by mixing 5 ml NZCYM overlay agar tempered at 45°C with 0.5 ml bacteria harvested in CBHI from Base II agar plates and incubated for 4 h as described above and poured onto NZCYM plates. Phage solutions were spotted manually with three times 10 μl of 10⁰–10⁻⁷ phage dilutions in SM buffer. Plates were incubated at 37°C for 20–24 h under microaerobic conditions and the number of plaque-forming units per milliliter (PFU/ml) of undiluted phage suspensions was calculated. Usually, phage titers of 10⁶–10⁸ PFU/ml could be obtained on both the propagating strains and *C. jejuni* MP21.

**PLAQUE ASSAY**

Susceptibility of a *C. jejuni* strain to the bacteriophages was performed using the plaque assay protocol described under Bacteriophage titration and determined as the efficiency of plaquing (EOP) in percent by dividing the PFU/ml on the test strain by the PFU/ml on the control strain (NCTC11168) multiplied by 100. The values represented are the mean count standard deviations derived from two to four independent experiments.

**HIGH-RESOLUTION MAGIC ANGLE SPINNING NMR SPECTROSCOPY**

High-resolution magic angle spinning (HR-MAS) NMR analysis of intact bacterial cells was performed as previously described (McNally et al., 2005).

**IN VIVO EXPERIMENTS**

One-day-old chickens were obtained from the Poultry Research Facility, Department of Agriculture, Food and Nutritional Science, University of Alberta. On the day of arrival, chickens were checked for *C. jejuni* colonization by cloacal swabs and divided in groups of four chickens. On day 2, chickens were first orally gavaged with either 10⁸ F336 phages in SM buffer or with SM buffer alone. After approximately 30 min, the chickens were orally gavaged with a suspension containing 10⁶ *C. jejuni* NCTC11168 in PBS or with PBS alone. Six days post infection, chickens were euthanized and the contents from one cecum were collected aseptically. The cecal contents were weighed and resuspended in phosphate-buffered saline at a final concentration of 0.1 g/ml. This suspension was subjected to 10-fold serial dilutions and plated onto Karmali selective agar plates to determine bacterial colonization levels. One hundred microliters of the 10⁻¹ dilution was plated separately onto selective plates and all resulting bacterial colonies were pooled together. Half of this pool was used for sequencing and phage sensitivity and the other half was used for NMR analysis. The experiment was repeated twice with consistent results. PCR of gene cj1421 was performed as previously described (Sørensen et al., 2011) while sequencing was done using the following primer: 1421-seq2-F (5’-CTCGAGTTATAAGGAATTTTAGATGAG-3’), Gene cj1426 was PCR-amplified using the primers: 1426-PCR-F (5’-TTGAGAATTGAAAGATGAGG-3’) and 1426-PCR-R (5’-TTTTCATTAGATCITTTCG-3’) and then sequenced using the primers: 1426-seq-F (5’-AACATCCAGATAAAAAGAGATGATTTGG-3’) and 1426-seq-R (5’-ATCAGAGAACATCGATTTTCGCC-3’).

**RESULTS**

**The MeOPN MOIETY OF THE CPS OF C. jejuni NCTC11168 IS A RECEPTOR FOR SEVERAL PHAGES**

We recently demonstrated that phage F336 requires the O-methyl phosphoramidate (MeOPN) moiety attached to the GalNAc of the *C. jejuni* NCTC11168 CPS for infection (Sørensen et al., 2011).
In our phage collection (Hansen et al., 2007) we found that five phages, including F336, rely on capsular moieties for infection of *C. jejuni* NCTC11168. In our previous study, we isolated a phage F336 resistant NCTC11168 variant in vitro (11168R), which lost the MeOPN attached to the GalNAc residue due to the presence of an additional G in gene cj1421 resulting in an off version of the gene (Sørensen et al., 2011). Here we found that 11168R had gained cross-resistance to all of the four phages: F198, F287, F303, and F326 (Table 3). While phages F198 and F303 did not form plaques on *C. jejuni* 11168R at all, plaques were observed with F287 and F326, but with a much lower EOP compared to wild type NCTC11168. Hence, the MeOPN structure is important for infection by the four other phages, although two of these phages (F287 and F326) were able to infect a strain not expressing the MeOPN modification. Similar results were obtained when screening a cj1421 MeOPN transferase deletion mutant in the NCTC11168 wild type background (data not shown). Supporting screening a number of times in our laboratory were analyzed by HR-MAS NMR and 1H–31P heteronuclear single-quantum correlation (HSQC) HR-MAS NMR to determine MeOPN and 6-O-Me groups (St Michael et al., 2002; Carrillo et al., 2004). Thus, several phages rely on the MeOPN moiety attached to the GalNAc residue of CPS for infection of *C. jejuni* NCTC11168.

### Table 3 | Efficiency of plaquing (EOP) of phages related to F336 as compared to NCTC11168.

<table>
<thead>
<tr>
<th>Phage</th>
<th>NCTC11168</th>
<th>11168R</th>
<th>S11168R</th>
<th>kpsM</th>
</tr>
</thead>
<tbody>
<tr>
<td>F198</td>
<td>100☆</td>
<td>75</td>
<td>82.5 ± 24.7</td>
<td>–</td>
</tr>
<tr>
<td>F287</td>
<td>100</td>
<td>0.04 ± 0.03</td>
<td>2300 ± 282.8</td>
<td>–</td>
</tr>
<tr>
<td>F303</td>
<td>100</td>
<td>–</td>
<td>350 ± 113.1</td>
<td>–</td>
</tr>
<tr>
<td>F326</td>
<td>100</td>
<td>0.02 ± 0.02</td>
<td>11.6 ± 6.7</td>
<td>–</td>
</tr>
<tr>
<td>F336</td>
<td>100</td>
<td>–</td>
<td>331.8 ± 64.3</td>
<td>–</td>
</tr>
</tbody>
</table>

☆EOP is calculated in percent as the PFU/ml of the phages on the test strain divided by the PFU/ml obtained on NCTC11168 multiplied by 100.

---

The MeOPN moiety of *C. jejuni* NCTC11168 can be attached to the GalNAc and the Hep residues in the CPS (McNally et al., 2007) and we speculated that the attachment site of MeOPN may be important for phage infection. We therefore conducted plaque assays and determined EOPs of the five phages F198, F287, F303, F326, and F336 on the hypermotel NCTC11168 variant 11168H as well as on defined mutants in the cj1421 and cj1422 MeOPN transferase genes in this background and compared to the NCTC11168 wild type strain. All of these strains have previously been characterized by HR-MAS NMR and 1H–31P heteronuclear single-quantum correlation (HSQC) HR-MAS NMR for the presence/absence of MeOPN in their CPS structure (McNally et al., 2007). Our results showed that the attachment site of MeOPN in the CPS clearly influenced the efficiency of the phages to infect a particular strain (Figure 1). In general, all phages showed a higher EOP when MeOPN was situated on the GalNAc residue (Figures 1A,E) as compared to MeOPN attached to the Hep residue in the CPS (Figure 1C). A lower EOP obtained with MeOPN attached to the Hep residue was further confirmed by screening a cj1421 + cj1422 deletion strain complemented with cj1422 (McNally et al., 2007) with our five phages (Figure 1F). Interestingly, the four phages F198, F287, F303, and F336 were not able to infect *C. jejuni* 11168H, which is the only strain expressing MeOPN on both GalNAc and Hep (Figure 1B), suggesting that two MeOPN moieties may inhibit phage infection. Finally, we observed that the presence of two other phase variable CPS modifications, the 3-O-Me and 6-O-Me groups, influenced the plaquing efficiency of the phages. We also isolated a 11168H single colony variant (11168H1) that by HR-MAS NMR and 1H–31P HSQC HR-MAS NMR analysis showed the same CPS profile as the NCTC11168 wild type strain (data not shown). The plake sensitivity profile observed with this strain (Figure 1G) confirmed the results obtained with NCTC11168 (Figure 1A). Thus, the presence of the 6-O-Me group on the CPS reduced the plaquing efficiencies of phages F287 and F326 (Figures 1A,E), suggesting that other phase variable CPS modifications besides MeOPN affect phage infection.

### C. jejuni PHAGES REQUIRE DIFFERENT COMBINATIONS OF PHASE VARIABLE CPS MODIFICATIONS FOR SUCCESSFUL INFECTION

Phase variable expression of the capsular modifications in *C. jejuni* NCTC11168 is well documented (Parkhill et al., 2000; St Michael et al., 2002; Szymanski et al., 2003). Three frozen stocks of NCTC11168 (MP24, MP25, and MP26) that had been passaged a number of times in our laboratory were analyzed by HR-MAS NMR and 1H–31P HSQC HR-MAS NMR analyses were performed to determine the CPS structure, i.e., the 3-O-Me, 6-O-Me, MeOPN-GalNAc, and MeOPN-Hep as compared to our NCTC11168 wild type (Table 4). We also included another NCTC11168 variant (V26) previously shown to have yet another different set of modifications (St Michael et al., 2002; Carrillo et al., 2004).

Plaque assays using our five phages showed that these *C. jejuni* strains had distinct phase sensitivity profiles (Figures 3B–E). Interestingly, *C. jejuni* MP26 that expresses both MeOPN moieties, similar to the phage-resistant *C. jejuni* 11168H (Figure 1B), could be infected by four of the five phages although with different efficiencies (Figure 3D). However, CPS of *C. jejuni* MP26 only contains the 3-O-Me group, whereas 11168H CPS contains both the 3-O-Me and 6-O-Me groups (Figures 1B and 3D). These results indicate that the presence of 6-O-Me may inhibit phage infection. This was further supported by the observation that none of the phages formed plaques when the 3-O-Me was exchanged with the 6-O-Me while retaining the MeOPN modification attached to GalNAc (Figures 3A,E). Furthermore, the presence of the 6-O-Me group in combination with a MeOPN situated on the GalNAc and a 3-O-Me group clearly inhibited infection by phage F287 and F326 (Figures 1E,G). Thus, our data suggest that the 6-O-Me group inhibits plaque formation in vitro and that all five phages require MeOPN attached to GalNAc together with the 3-O-Me to obtain the most efficient infection. However, different combinations of the phase variable CPS modifications appear to influence...
binding of the phages differently. In particular, phage F326 showed the lowest EOP values (Table 3; Figures 1 and 3) suggesting that other combinations of receptors, not investigated in this study, might be optimal for the binding of this phage.

**SELECTION FOR PHAGE-RESISTANT VARIANTS DURING C. jejuni COLONIZATION OF CHICKENS**

To assess whether C. jejuni is capable of becoming resistant to bacteriophage F336 in vivo during colonization of chickens, we orally gavaged 2-day-old chickens with F336 and subsequently with C. jejuni NCTC11168. After 6 days, the chickens were euthanized and the bacteria from the ceca were analyzed. We observed similar levels of colonization after 6 days of infection between the groups receiving bacteria only, as compared to those receiving bacteria and bacteriophages (i.e., 10⁹ CFU/g cecal content, Table 5). The bacteria grown from the cecal contents were directly pooled from plates and analyzed by HR-MAS NMR. All bacteria obtained from chickens infected with C. jejuni alone showed an NMR profile that was the same as the NMR spectrum of the inoculum: the bacteria expressed one MeOPN attached to GalNAc and the 3-O-Me group bound to Hep. However, bacteria from the group of chickens that received both bacteria and bacteriophages had lost the

![Figure 1](image-url)
Figure 2 | High-resolution magic angle spinning NMR spectra showing the differences in CPS modifications between NCTC11168 variants MP24, MP25 and MP26. ¹H CPMG spectra of intact C. jejuni cells. Abbreviations are: Hep, D-glycero-D-gluco-Hep; Rib, β-D-Ribf; GlcA, α-D-GlcA6(NGro); GalNAc, β-D-GalNAc; MeOPN, O-methyl phosphoramidate; OMe, O-methyl; NAc, N-acetyl; TSP, trimethylsilyl propionic acid standard.

Table 4 | ppm Values for ¹H HR-MAS NMR spectra for NCTC11168 variants and MeOPN transferase status.

<table>
<thead>
<tr>
<th>C. jejuni strain</th>
<th>Hep⁷</th>
<th>Rib</th>
<th>GlcA</th>
<th>GalNAc</th>
<th>3-O-Me</th>
<th>6-O-Me</th>
<th>NAc</th>
<th>MeOPN</th>
<th>cj1421/cj1422 (GalNAc/Hep)⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC11168 MP24</td>
<td>5.582</td>
<td>5.376</td>
<td>5.103</td>
<td>5.015</td>
<td>3.629</td>
<td>3.557</td>
<td>2.071</td>
<td>3.772</td>
<td>−/+</td>
</tr>
<tr>
<td>NCTC11168 MP25</td>
<td>5.599</td>
<td>5.383</td>
<td>5.148</td>
<td>5.039</td>
<td>−</td>
<td>3.569</td>
<td>2.068</td>
<td>(3.772)</td>
<td>−/−</td>
</tr>
<tr>
<td>NCTC11168 MP26</td>
<td>5.610</td>
<td>5.381</td>
<td>5.143</td>
<td>5.101</td>
<td>3.630</td>
<td>−</td>
<td>2.068</td>
<td>3.768/3.743</td>
<td>+/+</td>
</tr>
</tbody>
</table>

⁷Only anomeric proton resonances for carbohydrate residues are listed.
⁸Summary of sequencing results indicating the “on” (+) and “off” (−) status of the genes encoding the MeOPN transferases onto GalNAc (cj1421) and Hep (cj1422).
⁹Only trace amounts of MeOPN detected.

MeOPN in all instances, except for one (Figure 4; Table 5). In chicken #238, infected with phage F336 and C. jejuni, the bacteria showed reduced levels of MeOPN present on both GalNAc and Hep, but the bacteria also expressed high levels of 6-O-Me on the Hep (Figure 4).

To test whether the observed changes in CPS modifications resulted in phage resistance, we performed bacterial titration assays with all of the isolates recovered from the chickens. All isolates from the F336 infected birds had gained resistance to phage F336 and still showed similar levels of colonization (Table 5). Previously, we have shown that a loss of MeOPN on GalNAc can be the result of a switch in the polyG region of the MeOPN transferase gene cj1421 (Sørensen et al., 2011). To determine whether the loss of MeOPN and subsequent phage resistance were the result of a switch in the polyG region, we sequenced gene cj1421 from the pooled colonies from both experiments. All phage-sensitive isolates showed 9 G residues in the polyG region, while all phage-resistant isolates (except from chick 238) showed either 8 or 10 G residues in this region, which in both cases results in a non-functional transferase, due to a premature stop codon 1 or 5 amino acids after the polyG region, respectively (data not shown). Examination of the pooled colonies from chick 238 showed 9 G residues in the polyG region of cj1421, consistent with the observed MeOPN expression. However, sequencing of the polyG region of cj1426 from the pooled colonies from chick 238 showed 10 G residues indicating that the 6-O-Me transferase enzyme was functional (again consistent with the acquired expression of the Me modification in Figure 4) compared to 11 G residues in
FIGURE 3 | Capsular polysaccharide structures expressed by NCTC11168 variants and the corresponding phage sensitivity profiles. The CPS structure is schematically illustrated (modified from Guerry and Szymanski, 2008). Phage sensitivity patterns are calculated as the efficiency of plaquing (EOP) as compared to plaque formation on the NCTC11168 wild type strain.

<table>
<thead>
<tr>
<th>Phage</th>
<th>EOP on NCTC11168 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F198</td>
<td>100</td>
</tr>
<tr>
<td>F287</td>
<td>100</td>
</tr>
<tr>
<td>F303</td>
<td>100</td>
</tr>
<tr>
<td>F326</td>
<td>100</td>
</tr>
<tr>
<td>F336</td>
<td>100</td>
</tr>
</tbody>
</table>

We found that the phages F198, F287, F303, F326, and F336 all infected C. jejuni most efficiently when the 3-O-Me modification on Hep and a MeOPN moiety was present, with higher EOPs observed when the MeOPN modification was attached to Gal\(\text{NAc}\) rather than Hep. Interestingly, when the 3-O-Me was exchanged with the 6-O-Me while retaining the MeOPN on Gal\(\text{NAc}\), none of the phages formed plaques at all, demonstrating a clear role for the O-methyl Hep modifications for phage infection, in addition to the previously identified phage receptor, MeOPN (Sørensen et al., 2011). By looking at the predicted three dimensional (3D) structure of the C. jejuni NCTC11168 CPS (Guerry and Szymanski, 2008), it is possible that the presence or absence of one or more CPS modifications may lead to conformational changes that interfere with the phage binding process. Indeed it has been found that changes in the 3D conformation of the outer membrane protein OmpA, which serves as the receptor for many of the T-even phages infecting E. coli, leads to phage resistance (Riede and Eschbach, 1986). Furthermore, the spatial arrangement of the modifications may be important for phage recognition and some modifications may potentially block the phage binding site when the parental strain lacking this modification. Thus, C. jejuni can become resistant to phage F336 during in vivo colonization of chickens by changing the expression of its CPS phase variable modifications.

DISCUSSION

Implementation of efficient phage therapies to target pathogenic bacteria requires detailed knowledge of the bacterial receptors recognized by the phages, as this bacteria–phage interaction is the first event in a process leading to lysis of the host organism. We previously identified the C. jejuni NCTC11168 CPS phase variable MeOPN modification as the receptor for phage F336 (Sørensen et al., 2011). The goal of the present study was to further explore the role of MeOPN and other C. jejuni phase variable CPS modifications, such as O-methylation in phage infection. We compared five different phages, including F336 that were unable to proliferate in an acapsular mutant of C. jejuni NCTC11168, and determined their ability to infect NCTC11168 variants containing the same CPS backbone sugars as NCTC11168, but with different modifications.

A NCTC11168; (B) NCTC11168 MP24; (C) NCTC11168 MP25; (D) NCTC11168 MP26; and (E) NCTC11168 V26. *MeOPN only detected in minor amounts.

Abbreviations as above are: Gal\(\text{NAc}\), N-acetylgalactosamine in the furanose configuration; MeOPN, O-methyl phosphoramidate; Hep, heptose; and O-Me, O-methyl.
Table 5 | Presence of MeOPN and phage sensitivity of C. jejuni isolated after colonization of chickens.

<table>
<thead>
<tr>
<th>Infected with</th>
<th>Bacterial counts (CFU/g)</th>
<th>MeOPN on Gal/Nac</th>
<th>Phage sensitivity a</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP21 Inoculum</td>
<td>NR</td>
<td>NR</td>
<td>+</td>
</tr>
<tr>
<td>MP21 Karmali</td>
<td>NR</td>
<td>NR</td>
<td>+</td>
</tr>
<tr>
<td>Chick 405</td>
<td>MP21</td>
<td>$5.7 \times 10^9$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 406</td>
<td>MP21</td>
<td>$4.1 \times 10^9$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 407</td>
<td>MP21</td>
<td>$9.9 \times 10^8$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 408</td>
<td>MP21</td>
<td>$1.1 \times 10^9$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 505</td>
<td>MP21 + F336</td>
<td>$7.9 \times 10^8$</td>
<td>–</td>
</tr>
<tr>
<td>Chick 506</td>
<td>MP21 + F336</td>
<td>$5.0 \times 10^9$</td>
<td>–</td>
</tr>
<tr>
<td>Chick 507</td>
<td>MP21 + F336</td>
<td>$5.8 \times 10^9$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 508</td>
<td>MP21 + F336</td>
<td>$8.5 \times 10^8$</td>
<td>–</td>
</tr>
<tr>
<td>Chick 343</td>
<td>MP21</td>
<td>$2.0 \times 10^9$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 344</td>
<td>MP21</td>
<td>$2.1 \times 10^9$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 345</td>
<td>MP21</td>
<td>$1.6 \times 10^9$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 346</td>
<td>MP21</td>
<td>$3.6 \times 10^9$</td>
<td>+</td>
</tr>
<tr>
<td>Chick 237</td>
<td>MP21 + F336</td>
<td>$1.2 \times 10^9$</td>
<td>–</td>
</tr>
<tr>
<td>Chick 238</td>
<td>MP21 + F336</td>
<td>$5.5 \times 10^9$</td>
<td>+ b</td>
</tr>
<tr>
<td>Chick 239</td>
<td>MP21 + F336</td>
<td>$4.7 \times 10^9$</td>
<td>– c</td>
</tr>
<tr>
<td>Chick 240</td>
<td>MP21 + F336</td>
<td>$4.1 \times 10^9$</td>
<td>–</td>
</tr>
</tbody>
</table>

Chicks 405–408 and 505–508 are from the first experiment, while chicks 343–346 and 237–240 are from the second experiment.

aYes: plaques formed on isolated strain, No: no plaques formed on isolated strain.
bBacteria isolated from this chick showed reduced MeOPN levels (of both MeOPN groups) and enhanced levels of 6-O-Me.
cMeOPN levels barely above the baseline could be detected from bacteria isolated from this chick.

NR, not relevant.

they are expressed. Certainly masking the phage binding site is a well known resistance mechanism in other bacteria. One example is Staphylococcus aureus that masks its phage receptor with protein A, a cell-wall-anchored virulence factor for this bacterium (Nordstrom and Forsgren, 1974). In agreement with this hypothesis, we observed that when MeOPN was present on both the GalNac and Hep residues together with the 3-O-Me and 6-O-Me Hep modifications, then four of the five phages could no longer infect that particular C. jejuni 11168 strain. Thus, both the presence and absence of the capsular modifications appears to modulate phage infectivity of the NCTC1168 strain.

Our results demonstrate that the phages infecting C. jejuni are highly specific in the recognition of the CPS and that each phage has evolved to recognize a particular combination of capsular modifications. One might speculate that the phages included in this study originated from the same ancestral phage that has co-evolved with C. jejuni to recognize different capsular surface structures by minor modifications in the phage receptor binding proteins (RBPs). In support of this, sequencing of viral proteins from the C. jejuni phage CP220 revealed at least two distinct tail structures in the mature virions although propagated from a single plaque (Timms et al., 2010), suggesting a possibility for the phage to recognize multiple receptors. A similar phenomena has been observed for capsular phages infecting E. coli (Scholl et al., 2001). Indeed RBPs of other phages often have hypervariable regions in the receptor recognizing domains, reflecting the ability of the phage to easily accumulate mutations thereby changing the structure of the RBP to recognize modified receptors or new types of receptors, such as switching from protein to carbohydrate recognition (Montag et al., 1990; Drexler et al., 1991; Hashemolhosseini et al., 1994). The C. jejuni phages investigated in this study have similar genome sizes and morphologies and were previously isolated from duck and broiler intestines and duck abattoirs (Hansen et al., 2007). Although these phages all are able to infect the NCTC1168 strain, they do show different host ranges for C. jejuni strains of different Penner serotypes (Hansen et al., 2007). In addition, enzymatic restriction cleavage of the phage genomes revealed differences among the phages (Table 2, Hansen et al., 2007). Thus, although a conserved head and tail architecture is maintained, it is apparent that genomic dissimilarities exist between the five phages compared in this study and this may account for the differences in host specificity.

In C. jejuni, the CPS are highly diverse, due to differences in the genetic compositions of the CPS loci, but also because many of the genes in these loci contain homopolymeric tracts making them prone to phase variation (Linton et al., 2001; St Michael et al., 2002; Szymanski et al., 2003; Karlyshev et al., 2005; McNally et al., 2005; McNally et al., 2005; Chen et al., 2008). Based on the number of phase variable modifications in the capsule of C. jejuni NCTC11168 alone, this may result in the expression of >700 CPS structural variants. Our results demonstrate that phase variable CPS surface structures clearly influence the phage attachments sites in C. jejuni NCTC11168. Phase variable expression also affects phase adsorption in Bordetella spp. These bacteria use phase variable expression of the BvgAS two-component regulatory system to alter their surfaces through the regulation of colonization and virulence factors.
FIGURE 4 | NMR analysis of intact C. jejuni cells recovered from chicken cecal contents. (A) $^1$H HR-MAS NMR CPMG spectra, displaying the capsular profile and (B) corresponding $^{1}H$–$^{31}P$ HSQC spectra specifically demonstrating the phosphoramide resonances. Black arrowhead: note the change in the GalNAc resonance due to the absence of MeOPN. White arrowhead: note the absence of the MeOPN specific resonance.
and also expression of the phage BPP-1 receptor Prn (pertactin autotransporter; Liu et al., 2002). To combat these changes, the BPP-1 phage has a sophisticated mechanism of varying its RBP (~10^13 possible sequences) that approaches the levels of diversity observed for immunoglobulins (Liu et al., 2002; Doulatov et al., 2004), and the phage is thus able to overcome the resistance by an equally efficient mechanism.

It was previously shown that CPS expression is important for C. jejuni colonization of the chicken gut as well as being important for interactions with human models of disease (Bacon et al., 2001; Jones et al., 2004). Here we show for the first time that the presence of phages selects for specific CPS variants in vivo in the chicken gut without altering the colonization levels, even though MeOPN expression influences C. jejuni invasion of human cells (van Alphen, unpublished results). Thus, the roles of phase variable CPS modifications, such as the MeOPN and the O-methyl groups, may not only be linked to adaptation to the human or chicken hosts, but may serve an important function for C. jejuni survival in the avian gut; an environment that contains a high number of bacteria as well as phages (Connerton et al., 2004; Hansen et al., 2007). Scott et al. (2007) also described that certain isolates of C. jejuni containing Mu-like prophage DNA sequences were capable of developing bacteriophage resistance in the chicken gut through a mechanism of reversible genomic rearrangement. Remarkably, these isolates inverted genomic segments up to 590 kb which also led to a decrease in chicken colonization and production of a functional Mu-like bacteriophage (Scott et al., 2007).

The authors speculated that the development of phage resistance could be due to alterations in the bacterial surface receptor or through saturation of the receptor sites if the Mu phage is recognized by the same structures. Here, we have shown that phase variable modifications of the CPS influence phage infectivity, suggesting that the constant exposure of C. jejuni to phages in the avian gut selects for changes in the phase variable structures of the CPS leading to a continuing co-evolution of C. jejuni polysaccharides and phage RBPs. This should be considered when developing future strategies for phage therapy against C. jejuni in the chicken host and when determining the kinetics of resistance development and the timing for therapy administration. Since the alteration of bacterial surface receptors that reduces or eliminates phase adsorption to the host cell appears to be the most common mechanism of phage resistance (Bohannan and Lenski, 2000), continued investigations into the diversity of phage receptors in C. jejuni is required to select the most efficient combinations of phages for therapy.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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