Insect Infection Model for *Campylobacter jejuni* Reveals That O-methyl Phosphoramidate Has Insecticidal Activity

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*Galleria mellonella* (wax moth) larvae have elsewhere been shown to be susceptible to pathogens such as *Francisella tularensis*, *Burkholderia mallei*, and *Pseudomonas aeruginosa*. We report that the larvae are rapidly killed by *Campylobacter jejuni* at 37°C. Three strains of *C. jejuni* tested, 11168H (human diarrheal isolate), G1 (human Guillain-Barre syndrome isolate), and 81–176 (human diarrheal isolate), were equally effective at killing *G. mellonella* larvae. A panel of defined mutants of *C. jejuni* 11168H, in known or putative virulence genes, showed different degrees of attenuation in *G. mellonella* larvae. A mutant lacking the O-methyl phosphoramidate (OMePN) capsule side group was attenuated, clearly demonstrating that OMePN has a role in virulence. This new model of *C. jejuni* infection should facilitate the identification of novel virulence genes.

*Campylobacter jejuni* is the leading bacterial cause of gastroenteritis in the world, causing 2.4 million cases yearly in the United States alone and affecting 1% of the population, with poultry, milk, and water commonly implicated as sources or vehicles of infection [1]. In developing countries, *Campylobacter* is hyperendemic, a leading bacterial cause of diarrheal disease, and a major cause of infant mortality [2, 3]. Irritable bowel syndrome is a postinfectious complication of campylobacteriosis, compounding the economic burden of *C. jejuni* diarrheal disease [3, 4]. Furthermore, in some cases *C. jejuni* infection may result in complications such as meningitis, endocarditis, Reiter’s syndrome, and Guillain-Barré syndrome (GBS), including its variant, Miller Fisher syndrome [3].

Given the medical and socioeconomic importance of *Campylobacter* infection, it is remarkable that *C. jejuni* is one of the least understood enteropathogens. The identity of its core virulence determinants and its mechanisms of molecular pathogenesis have proved elusive. The absence of obvious enterotoxins or enterically active cytotoxins suggests that the organism causes diarrheaa in a manner quite distinct from that employed by well-established enteric pathogens, such as *Vibrio cholerae* and the toxigenic pathovars of *Escherichia coli*. The known cytolethal distending toxin remains enigmatic in terms of disease causation but, interestingly, may be a candidate for secretion via the flagellum [5]. Other identified virulence factors of *C. jejuni* include capsular polysaccharide [6], lipooligosaccharide (LOS) [7], and flagella [8].

There are 2 reasons for our poor understanding of *C. jejuni*. The first is a historical lack of molecular tools for *C. jejuni* genetic manipulation, which has now largely been overcome with the development of species-specific promoters and associated tools [9, 10]. The second reason is the lack of a suitable infection model.
**Table 1. Campylobacter jejuni Strains and Mutants Tested for Virulence in This Study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Description of strains and genes affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>11168H</td>
<td>[31]</td>
<td>Hypermotile variant of the sequenced strain NCTC11168</td>
</tr>
<tr>
<td>81–176</td>
<td>[32]</td>
<td>Human diarrheal isolate</td>
</tr>
<tr>
<td>G1</td>
<td>[33]</td>
<td>Isolate from patient with Guillain-Barré syndrome</td>
</tr>
<tr>
<td>11168H(cj)0079</td>
<td>A.V.K. and B.W.W.; unpublished data</td>
<td>Cytolethal distending toxin mutant</td>
</tr>
<tr>
<td>11168H(cj)0511</td>
<td>A.V.K. and B.W.W.; unpublished data</td>
<td>Gene cj0511 encodes product with sequence similarity to tail-specific proteases</td>
</tr>
<tr>
<td>LOS; 11168H(cj)1132–cj1152c deletion</td>
<td>[34]</td>
<td>Deletion of lipooligosaccharide biosynthesis region</td>
</tr>
<tr>
<td>11168H(cj)1228</td>
<td>A.V.K. and B.W.W.; unpublished data</td>
<td>Gene cj1228 product shows sequence similarity to HtrA protease (heat shock protein), found in many bacteria</td>
</tr>
<tr>
<td>11168H(cj)1321</td>
<td>[35]</td>
<td>Gene cj1321 is located in a flagella modification locus and encodes a putative transferase</td>
</tr>
<tr>
<td>11168H(cj)1324</td>
<td>[35]</td>
<td>Gene cj1324 is required for legionaminic acid modification of flagellin</td>
</tr>
<tr>
<td>11168H(cj)1339</td>
<td>[35]</td>
<td>Aflagellate</td>
</tr>
<tr>
<td>11168H(cj)1365</td>
<td>A.V.K. and B.W.W.; unpublished data</td>
<td>Gene cj1365 encodes a putative outer membrane protease (autotransporter protein)</td>
</tr>
<tr>
<td>11168H(cj)1413</td>
<td>[33]</td>
<td>Mutant cj1413 (kpsS) is deficient in capsule production</td>
</tr>
<tr>
<td>11168H(cj)1416</td>
<td>[36]</td>
<td>CPS produced in mutant cj1416 lacks MeOPN(^a) modification</td>
</tr>
<tr>
<td>11168H(cj)1416 cj1416(^a)</td>
<td>Current study</td>
<td>Complemented cj1416 mutant</td>
</tr>
</tbody>
</table>

\(^a\) MeOPN, O-methyl phosphoromimidate capsular polysaccharide

*C. jejuni* infection models previously used include a ferret diarrheal model [11], a colostrums-deprived piglet model [12], and a chick colonization model [13]. These have been used to identify potential virulence determinants [6, 8, 14, 15]. However, limitations associated with all of these models of infection, such as reproducibility, cost, ease of use, breeding, and specialized training, have precluded their widespread use [16]. In addition, because the avian gut is the normal site of carriage of the bacterium it is questionable whether avian colonization models are able to identify virulence mechanisms involved in human infection [3]. More recently, several transgenic mouse models have been reported, which have applications for understanding *C. jejuni* mechanisms of infection; these include limited-flora, interleukin 10–deficient, MyD88-deficient, and Nramp1\(^{-/}\) mice [17–20]. However, these models are expensive and not widely accessible to the research community. In addition, owing to genetic manipulations in mice that affect their immune status, the results must be carefully interpreted [19].

An alternative to mammalian models of infection is the use of invertebrate hosts, such as nematodes or insects. *Caenorhabditis elegans* has attracted attention as an infection model for a diverse range of bacterial pathogens, including *Burkholderia* species, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Yersinia pestis*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and the fungal pathogen *Cryptococcus neoformans* [21, 22]. However, *C. elegans* cannot survive at 37°C and lacks functional homologues of some components of the mammalian immune system, such as specialized phagocytic cells [23]. Furthermore, *C. elegans* dies rapidly in a microaerophilic atmosphere, the condition under which pathogens such as *Helicobacter pylori* and *C. jejuni* grow and survive. Models of infection based on insects, such as *Drosophila melanogaster*, *Galleria mellonella* larvae, and *Manduca sexta* larvae, offer the advantage that they can be used at 37°C. In addition, insects possess specialized phagocytic cells, also known as hemocytes or granulocytes [23]. These cells are able to phagocytose pathogens and kill them by using antimicrobial peptides and reactive oxygen species, which are generated during a respiratory burst [24, 25]. Therefore, insect hemocytes have many properties in common with mammalian phagocytes.

*G. mellonella* (wax moth) larvae have recently been shown to be susceptible to infection with pathogens, such as *Francisella tularensis* [26], *Burkholderia mallei* [27], *P. aeruginosa* [28], *C. neoformans* [29], and *Candida albicans* [30]. In this study we report that *G. mellonella* is susceptible to infection with *C. jejuni* and may provide a valuable model to elucidate virulence in this poorly understood pathogen.

**METHODS AND MATERIALS**

**Strains and culture conditions.** All bacterial strains and mutants used in this study are shown in Table 1. *C. jejuni* strain
11168H is a hypermotile variant of the sequenced strain NCTC11668 that readily colonizes chickens [31, 37]. C. jejuni strains were cultured on Columbia agar plates supplemented with either 5%–9% (vol/vol) horse blood or Skirrow selective supplement (Oxoid) and 5%–9% (vol/vol) horse blood in a variable-atmosphere incubator (Don Whitley Scientific) under microaerobic conditions (5% oxygen, 85% nitrogen, 10% carbon dioxide) at 37°C for 24 or 48 h. For some experiments, bacteria were cultured in sealed jars in an atmosphere of 6% oxygen and 10% carbon dioxide (CampyPak; Oxoid) for 48 h. Where necessary, Columbia agar plates were supplemented with antibiotics, kanamycin (50 μg/mL) and/or chloramphenicol (15 or 30 μg/mL).

For infections, bacteria were subcultured into 25 mL of Mueller-Hinton broth (Oxoid) and grown under microaerobic conditions as described above, for 24–48 h. The bacteria were then adjusted to an optical density at 590 nm (OD590) of 1.0 for infections. In some experiments bacteria were harvested from the plates into 1 mL of phosphate-buffered saline (PBS) and adjusted to an OD590 of 1.0. Infectious doses were confirmed by serially diluting the inocula and plating them onto Columbia agar supplemented with horse blood. Infections at lower doses were adjusted accordingly. Bacteria collected from the hemolymph of infected larvae were serially diluted in PBS and plated onto Columbia agar supplemented with 5% (vol/vol) horse blood and Campylobacter-selective supplement (Oxoid).

G. mellonella killing assays. Final instar G. mellonella larvae (Live Foods) were maintained on wood chips at 15°C. Larvae were infected with C. jejuni strains in 10-μL inocula by microinjection (Hamilton) in the right foreleg. The larvae were incubated at 25°C, 37°C, or 42°C, and survival and appearance were recorded at 24-h intervals. PBS-injected and un.injected controls were used. Survival 24 h after infection was recorded. To determine the numbers of bacteria and site of localization in the hemocoel, larvae were chilled on ice for 5 min. The rear 2 mm of each larva was aseptically removed, and the hemocoel was drained into a sterile 1.5-mL microcentrifuge tube. Bacteria were enumerated as described above. PBS-injected and un injected controls were used. Bacteria were heat killed at 95°C for 5 min, and sterility was checked by “streaking out.” For each experiment 10 G. mellonella larvae were infected, and experiments were repeated 3 times unless otherwise stated.

C. jejuni mutant construction. Mutants were constructed via insertion of the kanr cassette into unique restriction sites present in the gene fragments from a pUC18 library [36]. Gene cj1416 was inactivated via insertion of the kanr cassette into the PsI site of a fragment of this gene present in plasmid cam85c11. In order to mutate gene cj1324 the kanr cassette was inserted into an EcoRV site of plasmid cam19B. Derivatives with the correct orientation of the kanr cassette (colinear with the gene target) were selected by restriction analysis and used for transformation into C. jejuni strain 11168H. Allelic replacement, resulting in gene inactivation in Kan' clones selected after transformation, was confirmed by polymerase chain reaction (PCR) analysis using gene-specific and kanr-specific primers.

C. jejuni complementation. Complementation of mutant 11168H/cj1416 was achieved via introduction of an intact copy of gene cj1416 into an intergenic region within the rRNA gene cluster, as described elsewhere [36]. Briefly, the gene was PCR amplified by using primers ak296 (5′GAATAATTTAAAATGGATTCAATATCTTACGACGAG3′) and ak297 (5′STAAAGTCTCTATATTTCACATTTGCACCTCATAATCTC3′), and the product was digested by SmaI and XbaI restriction enzymes and cloned into SmaI/XbaI-digested vector pRRC for expression under the control of a constitutive camr promoter. The recombinant plasmid-containing camr-cj1416 cassette was transformed into mutant 11168H/cj1416, followed by selection of camr and kanr colonies. Integration of the gene cassette into the chromosome was confirmed by PCR, as described elsewhere [36].

RESULTS

Killing of G. mellonella by genetically diverse C. jejuni strains. To establish the relevance of G. mellonella as an infection model to screen for variations of virulence in wild-type strains of C. jejuni, we determined the lethality in G. mellonella of a panel of well-characterized C. jejuni strains: 11168H (human diarrheal isolate), G1 (human GBS isolate), and 81–176 (human diarrheal isolate) (Figure 1). Infection with approximately 10^6 colony-forming units (CFUs) of C. jejuni strain G1, which had been cultured on an agar plate at 37°C, resulted in a 25% average survival rate at 24 h after challenge. Similar doses (suspensions with an OD_{600} of 1.0) of C. jejuni strain 81–176 or

![Figure 1](https://academic.oup.com/jid/article-abstract/201/5/776/864580)
11168H resulted in average survival rates at 24 h of 43% and 36%, respectively. In later studies we found that the conditions under which C. jejuni strain 11168H was cultured affected virulence and mortality in G. mellonella larvae. Whereas some larvae survived a 10^6 CFU challenge if the bacteria were grown on agar at 37°C, all of the larvae were killed if C. jejuni were cultured at 37°C in broth. All of our subsequent experiments reported below were carried out with broth-grown C. jejuni.

**Dose-responsive killing of G. mellonella by C. jejuni strain 11168H.** The survival of G. mellonella larvae after injection of 1 ¥ 10^3, 1 ¥ 10^4, or 1 ¥ 10^6 CFU of C. jejuni 11168H and incubation at 37°C was monitored at 4 and 24 h after challenge. At the highest dose (1 ¥ 10^6 CFU), melanization of all of the larvae was observed at 4 h, although all the larvae were scored as alive. After challenge with 1 ¥ 10^3, 1 ¥ 10^4, or 1 ¥ 10^6 CFU, the percentage of surviving larvae at 24 h after challenge was 30%, 10%, or 0%, respectively. A distinctive black melanization was observed in all dead larvae (Figure 2). When 1 ¥ 10^6 CFU of C. jejuni 11168H were heat killed and then injected into G. mellonella larvae, no melanization of larvae and no deaths were observed up to 24 h after challenge. The injection of 1 ¥ 10^6 CFU of the related pathogen H. pylori (strain SS1 or 26696) did not result in the death of larvae under the same conditions (data not shown).

**Temperature-dependent monitoring of G. mellonella survival after infection with C. jejuni.** All of the G. mellonella larvae (n = 10) challenged with ~10^6 CFU of C. jejuni 11168H and incubated at 25°C or 37°C were dead at 24 h. Although all of the challenged larvae (n = 10) incubated at 42°C were dead at 24 h, the uninfect ed controls also died at this temperature. Therefore, this model may not be suitable to investigate genes that are expressed at 42°C (avian gut temperature).

**Variable levels of attenuation in C. jejuni 11168H mutants revealed by G. mellonella model.** To establish the relevance of G. mellonella as a C. jejuni virulence screen, larval survival was recorded after challenge with 10^6 CFU of a range of C. jejuni strains with gene insertional inactivations (Figure 3). All of the uninfected control larvae or larvae challenged with PBS were alive at 24 h, but none of the larvae challenged with C. jejuni 11168H survived. The mutants showed differing abilities to kill G. mellonella larvae. A flagella glycosylation mutant (cj1321) and a mutant that lacks legioniminic acid modification to the O-linked glycan of the flagella (cj1324) were not significantly attenuated compared with wild-type C. jejuni. The other mutants tested showed different degrees of attenuation. A mutant that lacked the LOS biosynthesis cluster (cj1132c to cj1152c) was the most highly attenuated mutant in G. mellonella.

**O-methyl phosphoramo tate capsule modification essential for virulence in G. mellonella model.** It is known that most C. jejuni strains have capsular polysaccharides with an unusual O-methyl phosphoromerase (MeOPN) modification. To investigate whether the MeOPN played a role in the virulence of C. jejuni, we challenged G. mellonella larvae with C. jejuni 11168H, mutant cj1416, which specifically lacks the MeOPN modification to the capsule and its complemented derivative, cj1416/cj1416+. After 24 h, all of the larvae challenged with the wild-type or cj1416-complemented strain had died, but all of the larvae challenged with Δcj1416 survived (Figure 4).

In a subsequent experiment, bacterial load was measured 20 min, 1 h, 4 h, or 24 h after challenge (Figure 5). Four hours after challenge, a reduction in bacterial load was observed in larvae infected with mutant cj1416, compared with wild-type and the complemented derivative cj1416/cj1416+. Because of larval death, the bacterial load of larvae infected with the wild-type or complemented cj1416 strain could not be measured 24 h after infection. However, no bacteria were recovered from larvae infected with mutant cj1416.

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**Figure 2.** Appearance of groups of 10 Galleria mellonella larvae 24 h after injection with 1 ¥ 10^3, 1 ¥ 10^4, or 1 ¥ 10^6 colony-forming units of broth-grown Campylobacter jejuni 11168H (shown left to right). Larvae were incubated at 37°C after challenge.

**Figure 3.** Ability of broth-grown Campylobacter jejuni mutants (10^9 colony-forming units) to kill Galleria mellonella at 37°C. Groups of 10 larvae were used, and the results shown are the means of 3 experiments recorded 24 h after challenge. Error bars indicate standard errors of the mean; PBS, phosphate-buffered saline. *P < .05 (survival compared with strain 11168H; Welch-corrected 2-tailed t tests for pooled data from 2 or 3 separate experiments).
Figure 4. Killing of Galleria mellonella larvae by broth-grown Campylobacter jejuni 1168H, mutant cj1416, which lacks the O-methyl phosphoramidate capsule modification and its complemented derivative (cj1416+). Groups of 10 larvae were used, and the results shown are the means of 3 experiments recorded at 24 h after challenge. Error bars indicate standard deviations; PBS, phosphate-buffered saline.

DISCUSSION

Although the currently available animal models of C. jejuni infection—including ferret diarrheal, colostrums-deprived piglet, and chick colonization models—have been used to identify determinants of virulence, their relevance to human disease is not clear. An additional limitation of these models is that they are unsuitable for screening of large numbers of mutants. Our results demonstrate that G. mellonella larvae can be a useful screen to identify potential virulence determinants. Insects and mammals share a common mechanism in their innate immune response to pathogens. Insect hemocytes, like mammalian neutrophils, phagocytose and kill pathogens through the production of superoxide. This homology may help explain the fate of pathogens during intracellular stages of their life cycle. However, the relevance of this model to human disease requires further investigation.

There are several advantages of G. mellonella larvae as an infection model. The larvae, which are the caterpillar stage of the wax moth, are commercially available and bred as live food for captive reptiles and amphibians. They are typically 1–2 cm long, are easy to handle, and survive for up to 3 weeks before pupating. During this time they do not require feeding and require minimal maintenance. Unlike many invertebrate models of infection, they can be given precise doses of bacteria by injection. This model can therefore demonstrate differences in virulence at 37°C, and it is well suited to screening large panels of C. jejuni isolates or mutants.

The infection of insects is accompanied by the generation of melanin [38]. This response is especially marked in G. mellonella larvae, which change in color from cream to pale or dark brown. The precise role of melanization in host defense is not known, but during this process melanin becomes deposited around pathogens [38].

In this study, we initially screened a panel of C. jejuni mutants for virulence in G. mellonella. Our results indicate that it is possible to discriminate attenuated mutants in G. mellonella and, moreover, to assess degrees of attenuation. We have found that mutants cj1321 and cj1324, which are defective in glycosylation of flagella, are not attenuated in G. mellonella. It was reported elsewhere that the flagellar glycosylation system plays a role in colonization of chickens [14], but the role of this system in human disease is not known.

The rapid death of G. mellonella larvae infected with C. jejuni suggested the role of a secreted protein in killing. Some of the genetically defined mutants in this study have been shown elsewhere to be attenuated in other infection models. For example, the cytolethal distending toxin (cj0079) has been shown to play a role in persistent infection and mucosal inflammation in susceptible mouse strains [39, 40], and we have shown that a cytolethal distending toxin mutant was attenuated in G. mellonella. The attenuation of an aflagellate mutant of C. jejuni (cj1339) in G. mellonella may reflect the secretion of cytolethal distending toxin through the flagella, which has been suggested elsewhere [5]. However, the attenuation we observed could also have arisen from the inability of the mutant to secrete other proteins via the flagellar apparatus [41]. Flagella-exported proteins include CiaB and FlaC, which play roles in colonizing host cells [41, 42], and FspA2, which promotes their apoptosis [43]. Our finding that an LOS mutant was completely attenuated in G. mellonella is consistent with the observation that phase variation of LOS affects the invasion of cultured cells [44]. However, our results are the first direct evidence that LOS...
is essential for virulence of \textit{C. jejuni}. The role of other genes, revealed by the testing of mutants in this study, provides new insight into their possible roles in virulence. For example, \texttt{cj0511} is defective in the production of a protease (A.V.K. and B.B.W., unpublished data) and our finding that it was markedly attenuated in \textit{G. mellonella} indicate that it merits further attention as a determinant of virulence.

Finally, we showed that a mutant of \textit{C. jejuni} defective in the production of capsular polysaccharide was markedly attenuated in \textit{G. mellonella}. A mutant lacking a specific MeOPN modification of the \textit{C. jejuni} capsule was attenuated, and virulence was fully restored on complementation. This modification is found in many but not all strains of \textit{C. jejuni} and is especially associated with isolates from patients with enteritis, GBS, or Miller Fisher syndrome [36]. All 3 wild-type strains tested in this study possess the MeOPN modification to the capsule [36]. The structure of the MeOPN moiety shows remarkable similarities to the active structures of organophosphorous pesticides. The killing of \textit{G. mellonella} larvae may be a consequence of the toxicity provided by the MeOPN.

In summary, research into \textit{C. jejuni} pathogenesis has lagged behind that for other enteric pathogens, owing to the lack of an easily available, reliable infection model to identify determinants of virulence. \textit{G. mellonella} has been shown to be susceptible to infection by a number of bacterial pathogens. The aim of this research was to determine whether \textit{G. mellonella} could be used as an in vivo model to screen for \textit{C. jejuni} virulence determinants. We have clearly demonstrated that wild-type \textit{C. jejuni} actively kills \textit{G. mellonella} in a dose-responsive manner. We have shown that \textit{C. jejuni} strains lacking well-characterized virulence factors, such as LOS, cytolethal distending toxin, or motility, are significantly attenuated in \textit{G. mellonella}. Furthermore, we have shown that 2 putative \textit{C. jejuni} virulence determinants, a putative protease and the MeOPN capsule modification, are virulence factors in \textit{G. mellonella}. Understanding the role of MeOPN in human disease and its insecticidal activity will require further investigation.

Taken together, these results indicate that \textit{G. mellonella} is a suitable infection model for identification of \textit{C. jejuni} virulence determinants. A reliable, cheap \textit{C. jejuni} infection model would reduce dependence on mammalian and poultry infection models and have several applications for the Campylobacter research community; the ability to distinguish between virulent and avirulent \textit{C. jejuni} isolates would aid the identification of putative virulence genes through comparative genomics studies. Moreover, identification of virulent isolates and the ability to track them in animal or clinical populations would inform epidemiological studies and biocontrol approaches. Work is currently in progress to further develop \textit{G. mellonella} as an infection model for \textit{C. jejuni} and explore its relevance to human disease.

References

13. Wassenaar TM, van der Zeijst BA, Ayling R, et al. Identification of virulent isolates and the ability to track them in animal or clinical populations would inform epidemiological studies and biocontrol approaches. Work is currently in progress to further develop \textit{G. mellonella} as an infection model for \textit{C. jejuni} and explore its relevance to human disease.


