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CapC, a Novel Autotransporter and Virulence Factor of *Campylobacter jejuni*

Jai W. Mehat,[a] Simon F. Park,[b] Arnoud H. M. van Vliet,[a] Roberto M. La Ragione[a]

[a]Department of Pathology and Infectious Diseases, School of Veterinary Medicine, University of Surrey, Guildford, United Kingdom

[b]Department of Microbial Sciences, School of Biosciences and Medicine, University of Surrey, Guildford, United Kingdom

ABSTRACT *Campylobacter jejuni* is recognized as an important causative agent of bacterial gastroenteritis in the developed world. Despite the identification of several factors contributing to infection, characterization of the virulence strategies employed by *C. jejuni* remains a significant challenge. Bacterial autotransporter proteins are a major class of secretory proteins in Gram-negative bacteria, and notably, many autotransporter proteins contribute to bacterial virulence. The aim of this study was to characterize the *C. jejuni* 81116 C8J_1278 gene (*capC*), predicted to encode an autotransporter protein, and examine the contribution of this factor to virulence of *C. jejuni*. The predicted CapC protein has a number of features that are consistent with autotransporters, including the N-terminal signal sequence and the C-terminal β-barrel domain and was determined to localize to the outer membrane. Inactivation of the *capC* gene in *C. jejuni* 81116 and *C. jejuni* M1 resulted in reduced insecticidal activity in *Galleria mellonella* larvae. Furthermore, *C. jejuni capC* mutants displayed significantly reduced adherence to and invasion of nonpolarized, partially differentiated Caco-2 and T84 intestinal epithelial cells. Gentamicin treatment showed that the reduced invasion of the *capC* mutant is primarily caused by reduced adherence to intestinal epithelial cells, not by reduced invasion capability. *C. jejuni capC* mutants caused reduced interleukin 8 (IL-8) secretion from intestinal epithelial cells and elicited a significantly diminished immune reaction in *Galleria* larvae, indicating that CapC functions as an immunogen. In conclusion, CapC is a new virulence determinant of *C. jejuni* that contributes to the integral infection process of adhesion to human intestinal epithelial cells.

IMPORTANCE *Campylobacter jejuni* is a major causative agent of human gastroenteritis, making this zoonotic pathogen of significant importance to human and veterinary public health worldwide. The mechanisms by which *C. jejuni* interacts with intestinal epithelial cells and causes disease are still poorly understood due, in part, to the heterogeneity of *C. jejuni* infection biology. Given the importance of *C. jejuni* to public health, the need to characterize novel and existing virulence mechanisms is apparent. The significance of our research is in demonstrating the role of CapC, a novel virulence factor in *C. jejuni* that contributes to adhesion and invasion of the intestinal epithelium, thereby in part, addressing the dearth of knowledge concerning the factors involved in *Campylobacter* pathogenesis and the variation observed in the severity of human infection.

KEYWORDS *Campylobacter*, autotransporter proteins, virulence determinants

Campylobacter jejuni is a Gram-negative, microaerophilic bacterium that is recognized as the foremost cause of bacterial foodborne gastroenteritis worldwide (1). Infection is often self-limiting and is frequently acquired through the consumption of

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Address correspondence to Jai W. Mehat, jw.mehat@surrey.ac.uk, or Roberto M. La Ragione, r.laragione@surrey.ac.uk.

contaminated poultry and poultry products. It is estimated that *Campylobacter* causes 1.3 million cases of food poisoning in the United States and 280,000 cases in the United Kingdom each year (2). Moreover, campylobacteriosis poses a heavy economic burden, costing the U.S. economy an estimated $1.7 billion per annum and the U.K. economy an estimated £900 million per annum (3, 4).

The availability of a wealth of genomic information and the development of molecular tools for the study of *Campylobacter* has led to the identification of several factors integral to human infection and chicken colonization (5–7).

C. jejuni transiently colonizes the human intestinal tract, causing a mild watery diarrhea but can also cause a more severe inflammatory and bloody diarrhea (7–10). These diverse clinical manifestations may reflect differences in the virulence potential of *C. jejuni* isolates. The processes of adhesion to, invasion of, and translocation across the intestinal epithelium have been shown to be essential requirements to determine the pathogenicity of *C. jejuni*, yet the mechanisms *C. jejuni* uses for adhesion and invasion and whether these processes are intrinsically related are not entirely understood (11, 12). *C. jejuni* isolates have been shown to vary considerably in their abilities to both adhere and invade intestinal epithelial cells (13, 14). There is also a degree of variation reported in the translocation potential of *C. jejuni* and inconsistencies as to the route by which *C. jejuni* crosses the epithelium (15). Furthermore, different *C. jejuni* genotypes vary in their infection biology of the chicken host (13, 16); these inconsistencies may reflect various infection capabilities of different genotypes.

In excess of 40 genetic factors have been reported to contribute to *C. jejuni* adhesion and invasion (17). The precise influence and relative importance of each of these pathogenicity-associated factors to adhesion, invasion, and translocation of *C. jejuni* at the epithelial interface are unknown however. A clearer understanding of all of the factors contributing to this interaction is required to clarify the events during *C. jejuni* adhesion to and invasion of enterocytes in order to understand variation in virulence and clinical manifestation.

Bacterial autotransporters are the largest and most diverse class of surface-exposed or secreted proteins in Gram-negative bacteria (18, 19). These proteins share a mechanism of export, conferred by their C-terminal β-barrel structure, and play a wide variety of roles in pathogenesis and virulence conferred by their N-terminal functional or "passenger" domain (20). Significantly, all characterized autotransporter proteins are implicated in virulence to some extent (18). Notable autotransporters include the following: the *Bordetella pertussis* protein pertactin, a key component of epitopes included in the whooping cough vaccine (21); Yap proteins in *Yersinia pestis* and VacA; and the pore-forming, vacuolating cytotoxin in *Helicobacter pylori* (18).

The *capA* (*cj0628/9*) and *capB* (*cj1677/8*) genes of *C. jejuni* NCTC 11168 were the first two autotransporters identified in *C. jejuni* (22). CapA has been shown to contribute to adherence to Caco-2 cells and chicken LHM cells, and the absence of CapA has been shown to contribute to impaired colonization of chickens (22). However, a separate study concluded that CapA had no effect on broiler colonization (5). Furthermore, the *capA* gene is not present in all *C. jejuni* isolates, so it is likely dispensable (5), and no discernible role has been ascribed to CapB (5). These autotransporters are absent in *C. jejuni* M1 and *C. jejuni* 81116 (NCTC 11828), two other reference strains that were isolated from chickens and humans, respectively (23–25). In the absence of *capA* and *capB*, these strains can be expected to carry genes that encode other genetic factors to compensate.

Here, we characterized another putative autotransporter-encoding gene, the *C. jejuni* 81116 C8J_1278 locus (26), which bears no sequence identity with other autotransporters identified in *C. jejuni* and this autotransporter has been tentatively named CapC. Given the plethora of literature citing the involvement of autotransporters in virulence (18, 22, 27–30), we hypothesized that CapC serves as a virulence determinant in *C. jejuni*, and here, we show data supporting this hypothesis.

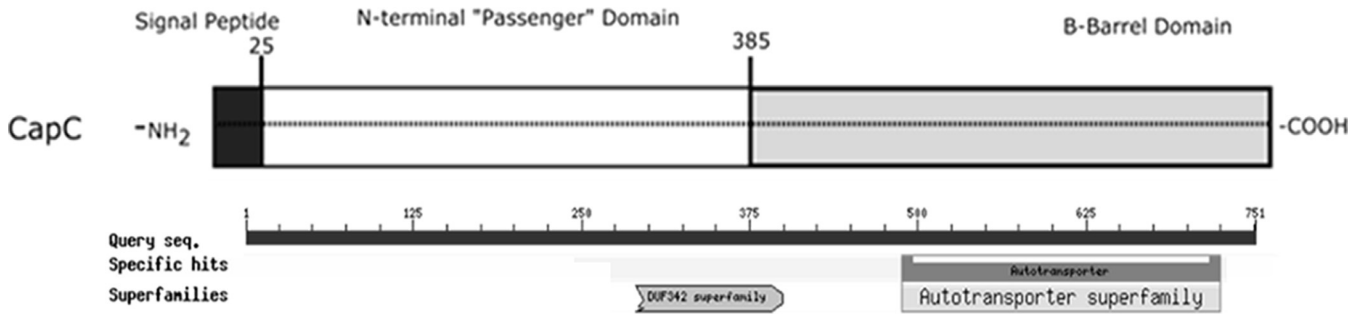

FIG 1 Analysis of CapC domains using the Conserved Domain Database query. The C-terminal third of the CapC protein sequence is shown to possess an autotransporter domain, confirming that CapC is a member of the autotransporter family. Query seq., query sequence.

RESULTS

Characterization of a novel *Campylobacter* autotransporter protein. The predicted protein sequence encoded by *capC* (GenBank accession no. ABV52877.1) was used to identify potential conserved domains using the NCBI Conserved Domain Database (CDD). The CDD query tool allowed the identification of structural motifs in the C-terminal thirds of the CapC sequence (residues 490 to 750). The CDD search successfully identified an autotransporter beta-domain in this region that was found in all autotransporter proteins, thus denoting CapC as a member of this family (Fig. 1). The SignalP 4.1 server was successfully utilized to predict the presence and location of a signal peptide sequence motif in the CapC amino acid sequence. The first 25 amino acids in the CapC sequence represent the N-terminal signal sequence of this autotransporter, and this sequence is cleaved after this residue. The subcellular localization prediction tool Cello V2.5 is commonly used to predict the localization of prokaryotic and eukaryotic proteins. Use of the Cello localization tool suggests that CapC is ultimately an extracellular, secreted protein. An alternative localization prediction tool is PSORTb. CapC is predicted by PSORTb to be primarily an outer membrane protein, as one might expect from an autotransporter; however, there was a lower prediction score for the likelihood of CapC being an extracellular protein. Phyre2 was successfully used in this study to build a partial three-dimensional (3D) model of the CapC protein based on remote homology detection methods. The model of the CapC protein structure shows the clearly defined 14 amphipathic strands comprising the beta-barrel that is characteristic of all autotransporters, further confirming CapC as a member of this family. Outputs from these analyses are shown in Fig. S1A to S1C and Table S1 in the supplemental material (31–34).

***capC* encodes an outer membrane protein that does not affect *C. jejuni* growth.** Antibodies against a CapC-specific peptide were raised in rabbits. Crude rabbit sera recognized an ~43-kDa protein in whole-cell preparations (Fig. 2A). Interestingly, an additional protein band was detected at ~25 kDa, which may represent a folded form of CapC. Only the ~43-kDa CapC protein was detected in the outer membrane fractions of wild-type *C. jejuni* 81116 and *C. jejuni* M1 (Fig. 2B and C), indicating that CapC is an outer membrane-anchored protein and does not appear to be secreted extracellularly in its native form. Anti-major outer membrane protein (anti-MOMP) antibodies were used as positive controls and to ensure purity of outer membrane fractions (Fig. S2) (35).

To assess the role of CapC in *C. jejuni*, isogenic mutants of *capC* were constructed in *C. jejuni* 81116 and *C. jejuni* M1 by insertion of a chloramphenicol resistance cassette. We were unable to complement the *capC* mutants, as we were unable to clone the *capC* coding sequence into the pC46 or pSV009 complementation vector in the correct orientation, despite repeated attempts (36–38).

The *capC* mutants lacked expression of CapC as determined by immunoblotting (Fig. 2A), and inactivation of *capC* did not affect growth of *C. jejuni* or reduce capability to survive exposure to aerobic conditions (Fig. S3 and S4).

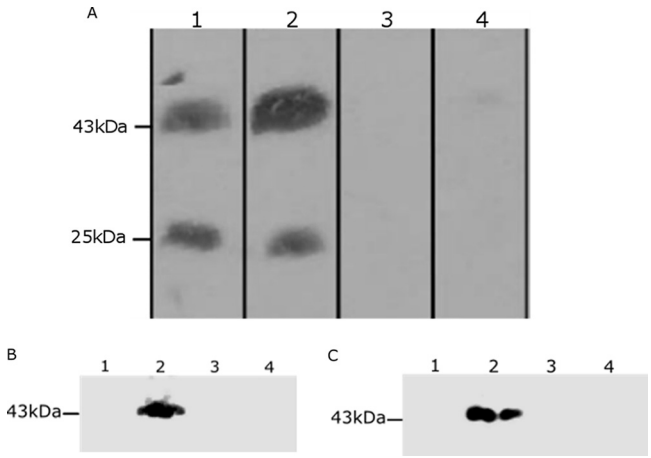

FIG 2 Immunodetection of CapC using anti-CapC rabbit primary antibody and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP). (A) Lane 1, *C. jejuni* 81116 whole-cell lysate; lane 2, *C. jejuni* M1 whole-cell lysate; lane 3, *C. jejuni* 81116 *capC* mutant whole-cell lysate; lane 4, *C. jejuni* M1 *capC* mutant whole-cell lysate. (B and C) Wild-type *C. jejuni* 81116 (B) and wild-type *C. jejuni* M1 (C) cellular fractions. Lanes 1, concentrated supernatant fraction; lanes 2, outer membrane fraction; lanes 3, inner membrane fraction; lanes 4, cytoplasmic fraction.

Inactivation of *capC* causes decreased virulence in the *Galleria mellonella* insect model. A larval survival assay and a morbidity and mortality screen were employed to assess the contribution of CapC to virulence. Larval survival levels were used as an indicator of virulence. The proportion of surviving larvae infected with mutant strains was significantly ($P < 0.05$) increased compared to wild-type strains from 6 to 48 h postinfection (Fig. 3). Mock-infected larvae showed 100% survival rates over 48 h. These results suggest that inactivation of *capC* causes decreased *C. jejuni* virulence in the *Galleria* model.

The contribution of *capC* to morbidity and mortality in the *Galleria* model was also assessed over a period of 24 h postchallenge on the basis of their ability to move, melanization and death using the scoring system outlined in Table S3. *C. jejuni* 81116 and *C. jejuni* M1 *capC* mutant strains exhibited significantly decreased morbidity and mortality in the *Galleria* model compared to parental strains based on all criteria (Fig. 4A and B). Wild-type strains consistently elicited mortality rates of 90 to 100%, whereas *C. jejuni* 81116 *capC* caused 10 to 33% mortality (q value [false-discovery rate] of <0.001) and *C. jejuni* M1 *capC* mutant caused 0 to 57% mortality (q value of <0.001). Notably, deletion of *capC* did not result in a complete cessation in virulence. *Galleria mellonella* larvae exhibit discoloration upon infection, which is an immune response that results in the deposition of melanin to encapsulate pathogens. *capC* mutants caused significantly decreased melanization (q value of <0.001), suggesting that CapC is an immunogenic factor that contributes to the inflammatory response *in vivo*.

CapC is required for maximal adhesion to Caco-2 and T84 intestinal epithelial cells. In order to further investigate the virulence phenotype with which CapC has been correlated, a combination of Caco-2 colorectal epithelial cells and T84 colonic epithelial cell culture models were used. Figure 5A shows adhesion and invasion efficiencies of *C. jejuni* 81116 and *C. jejuni* M1 and their respective *capC* mutants in a partially differentiated Caco-2 cell model, while Fig. 5B shows adhesion and invasion efficiencies of *C. jejuni* 81116 and *C. jejuni* M1 and their respective *capC* mutants in a T84 cell model. Inactivation of *capC* resulted in significantly reduced adherence and invasion of nonpolarized partially differentiated Caco-2 cells relative to wild-type strains ($P = 0.006$).

The *capC* mutants also show reduced adhesion and invasion efficiency in a T84 nonpolarized adhesion and invasion model; although this reduction was not statistically significant in the T84 cell culture model ($P = 0.06$), it was a consistent phenotype observed for both *C. jejuni* 81116 and *C. jejuni* M1.

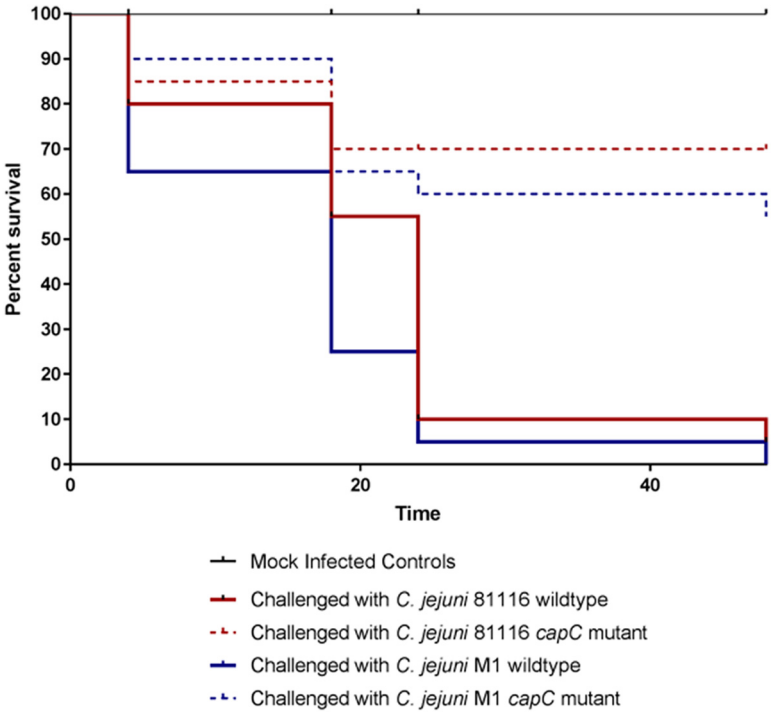

FIG 3 Survival of *Galleria mellonella* challenged with *C. jejuni* strains. The larval survival assay was performed with *C. jejuni* 81116, *C. jejuni* M1, and their respective *capC* mutants. Time (in hours) is shown on the *x* axis. Mock-infected controls treated with PBS were also used. Deletion of *capC* in both *C. jejuni* 81116 and *C. jejuni* M1 causes increased survival of *Galleria* larvae over 48 h. This decrease in survival becomes apparent as early as 4 h postinfection with the onset of larval death which decreases further thereafter. Comparison of mean survival at 24 h postinfection shows that deletion of *capC* causes significantly decreased survival (*C. jejuni* 81116, $P < 0.05$; *C. jejuni* M1, $P < 0.05$). These assays were performed in duplicate (10 larvae per replicate) on three different occasions. *P* values were obtained using an unpaired *t* test.

In order to assess whether inactivation of *capC* affected invasion or indirectly via reduced adherence, the number of *C. jejuni* surviving gentamicin treatment was represented as a proportion of the number of total associated bacteria (from which the number of adherent bacteria are calculated) and compared (Fig. 5C). The *capC* mutant strains show a slight increase in the proportion of invaded bacteria compared to their coupled wild-type strains, demonstrating that CapC does not cause a proportional decrease in invasion capacity. Therefore, results from adhesion and invasion assays using Caco-2 and T84 cell lines indicate that CapC directly impacts upon adhesion of *C. jejuni* 81116 and *C. jejuni* M1 *in vitro*, thereby subsequently reducing invasion efficiency.

CapC contributes to IL-8 secretion but does not affect cytotoxicity. Wild-type *C. jejuni* and *capC* mutant strains were compared for cytotoxic activity against Caco-2 cells (Fig. S5). *C. jejuni* 81116 elicited marginally greater cytotoxicity than its *capC* mutant counterpart, though this difference was not found to be statistically significant ($P = 0.09$). The difference in cytotoxicity elicited between *C. jejuni* M1 and *C. jejuni* M1 *capC* mutant was minimal ($P = 0.389$). These data suggest that CapC does not mediate cytotoxicity and that the levels of cytotoxicity differ markedly between *C. jejuni* strains.

Interleukin 8 (IL-8) is functionally analogous to insect chemoattractants, such as plasmatocyte spreading peptide (PSP), that promote hemocyte migration and melanin deposition in *Galleria* larvae and other insects (39). Given that *capC* gene deficiency caused decreased melanization, presumably through a lack of stimulation of the immune system of *Galleria*, we aimed to investigate whether *capC* gene deficiency also caused decreased activation of IL-8. The *capC* mutants of *C. jejuni* 81116 and *C. jejuni* M1 induced a decreased IL-8 response from T84 intestinal epithelial cells compared to respective wild-type strains (Fig. 6). This was more pronounced with *C. jejuni* M1

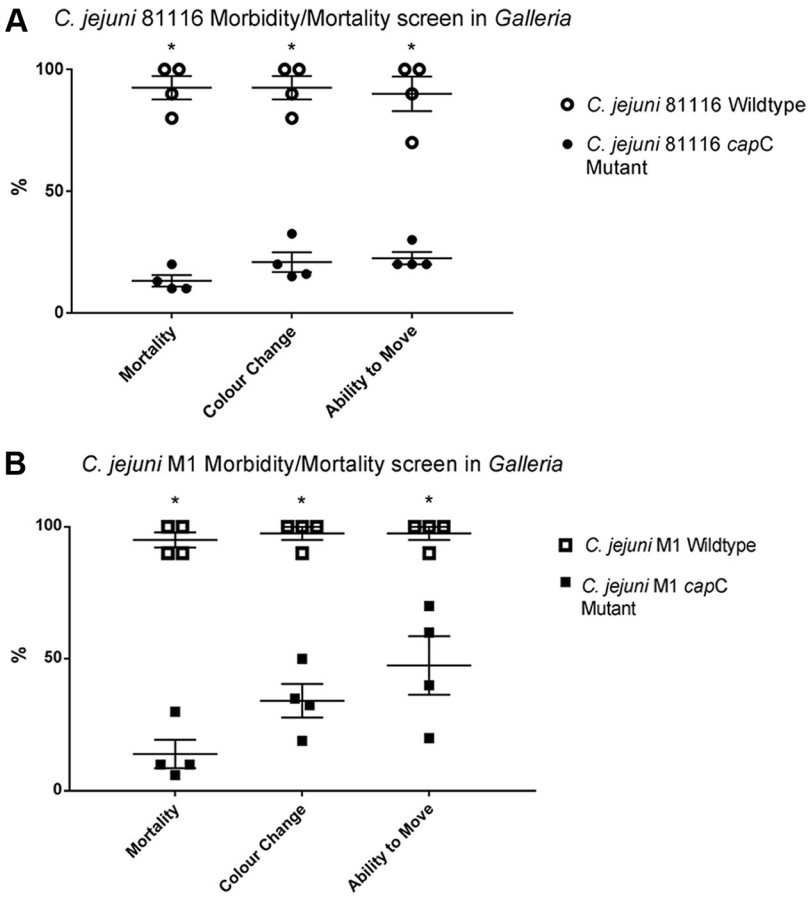

FIG 4 Morbidity and mortality of *Galleria mellonella* infected with *C. jejuni* 81116 (A), *C. jejuni* M1 (B) and their respective *capC* mutants. Twenty-four hours postinfection (p.i.), larvae were assessed for mortality, discoloration, and ability to move and assigned a score based on the scoring system in Table S4 in the supplemental material. Mean virulence scores of biological replicates for each criterion were converted to percentages of the maximum possible score and compared. There were significant differences (q value of <0.001) in the morbidity and mortality elicited by *C. jejuni* 81116 and its *capC* mutant and in the morbidity and mortality elicited by *C. jejuni* M1 and its *capC* mutant as indicated by the asterisks. Morbidity and mortality scores were compared using the multiple t test function in Graphpad Prism v7.03. These assays were performed in triplicate (10 larvae per replicate) on four different occasions; error bars show standard errors of the means.

compared to *C. jejuni* 81116. The *capC* mutant shows a greater reduction in IL-8 secretion ($P < 0.05$) than the wild-type *C. jejuni*. These phenotypes are reflective of adhesion and invasion phenotypes observed in the T84 cell model. While the magnitude of the decrease in IL-8 secretion from T84 cells is not consistent in the two *capC* mutant strains, the observed decrease indicates that CapC may play a role in eliciting immune responses from human intestinal epithelial cells.

DISCUSSION

During infection, damage to the human gut is mediated by *C. jejuni* adhesion to, invasion, and translocation across the epithelium (8, 14, 40). Many *Campylobacter* genes have been proposed to be involved in this interaction, and a variety of mechanisms by which these events occur have been reported (8, 14, 40–42). These genes include the genes encoding fibronectin binding proteins CadF and FlpA (5, 43), flagellar genes (44), and genes encoding surface structures such as capsule biosynthesis genes, lipooligosaccharide synthesis (LOS) and capsular polysaccharide synthesis (CPS) genes (15, 45). A number of metabolism-related genes have also been reported to be involved in adhesion and invasion, though these may merely be required for survival of *C. jejuni* during infection (46, 47). Furthermore, the host receptors, kinases, and pathways

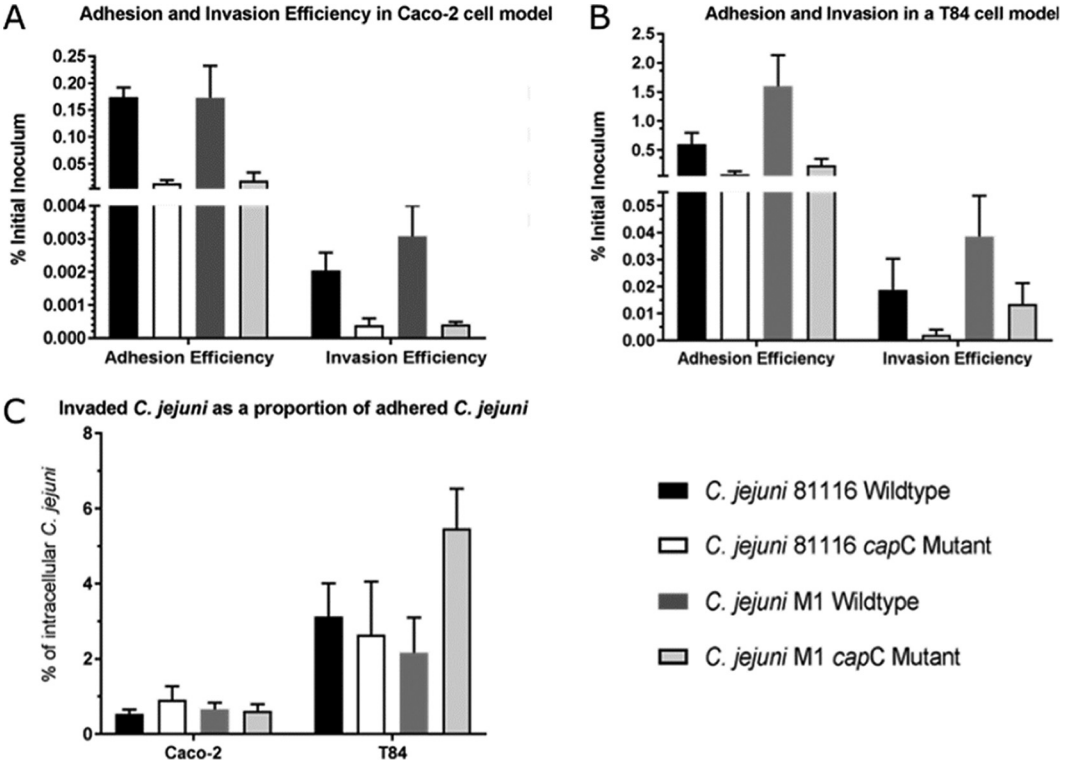

FIG 5 Adhesion and invasion capability of wild-type *C. jejuni* and *capC* mutant with intestinal epithelial cells. (A) Adhesion and invasion efficiencies of *C. jejuni* 81116, *C. jejuni* M1, and *capC* mutants in a Caco-2 cell culture model infected at an MOI of 1:200. *C. jejuni* 81116 exhibited an adhesion efficiency of 0.174%, whereas the *capC* mutants exhibited an adhesion efficiency of 0.014% ($P = 0.0067$). Wild-type *C. jejuni* M1 demonstrated a mean adhesion efficiency of 0.173%, whereas the *C. jejuni* M1 *capC* mutant exhibited an adhesion efficiency of 0.0188% ($P = 0.033$). The wild-type *C. jejuni* 81116 strain exhibited greater invasion efficiency (0.002%) than the mutant strain (0.00039%) ($P = 0.048$). *C. jejuni* M1 *capC* mutant had a significant impairment in its ability to invade Caco-2 cells compared to the wild-type strain ($P = 0.03$). These assays were performed in triplicate on three different occasions; error bars show standard errors of the means. (B) Adhesion and invasion efficiencies of *C. jejuni* 81116, *C. jejuni* M1, and *capC* mutants in a nonpolarized T84 cell culture model infected at an MOI of 200. Wild-type *C. jejuni* 81116 and wild-type *C. jejuni* M1 demonstrate adhesion efficiencies of 0.59% and 1.6%, respectively, compared to 0.078% and 0.24% for their *capC* mutants ($P > 0.05$). *C. jejuni* 81116 invasion efficiency was reduced from 0.019% to 0.002% as a result of *capC* inactivation, and *C. jejuni* M1 invasion efficiency was reduced from 0.039% to 0.014% ($P > 0.05$). Assays were performed in triplicate on three different occasions; error bars show standard errors of the means. (C) Proportion of associated *C. jejuni* that survive gentamicin treatment in a Caco-2 cell model and a T84 cell culture model. The viable count (in CFU per milliliter) of *C. jejuni* surviving gentamicin treatment is used to calculate invasion efficiency. Values shown are calculated as the number of invaded bacteria divided by the number of associated bacteria and converted to a percentage. The *capC* mutant strains show a slight increase in the proportion of invaded bacteria compared to their coupled wild-type strains, indicating that CapC likely does not cause a proportional decrease in invasion capacity (*C. jejuni* 81116, $P > 0.999$ by Mann-Whitney test; *C. jejuni* M1, $P > 0.999$) by Mann-Whitney test. Error bars show standard errors of the means.

involved in *C. jejuni* binding and cell entry are not very well characterized (14). Autotransporters are a class of secretory proteins in Gram-negative bacteria, all of which possess a unifying structure; a single polypeptide composed of an N-terminal signal peptide and passenger domain, which confer targeting to the inner membrane and a virulence function, respectively (18–20, 29). The C-terminal β-barrel domain, or autotransporter domain, is thought to form a pore in the outer membrane through which the passenger domain is secreted (20). The only autotransporters identified in *C. jejuni* thus far are CapA, CapB, and an additional autotransporter encoded by the *CJ81176_1367* locus in *C. jejuni* 81-176 (22, 48). CapA is an autotransporter that was previously shown to function as an auxiliary adhesin and may contribute to chicken colonization (22). The role of autotransporters in Gram-negative pathogens is diverse and not limited to adhesion to host cells; these proteins commonly function as serine proteases, esterases, mucinases, toxins, and hemagglutinins (18). In this study, we have characterized a novel autotransporter in *C. jejuni*, which we have termed CapC. Bioinformatic analysis has revealed that CapC possesses a number of molecular features

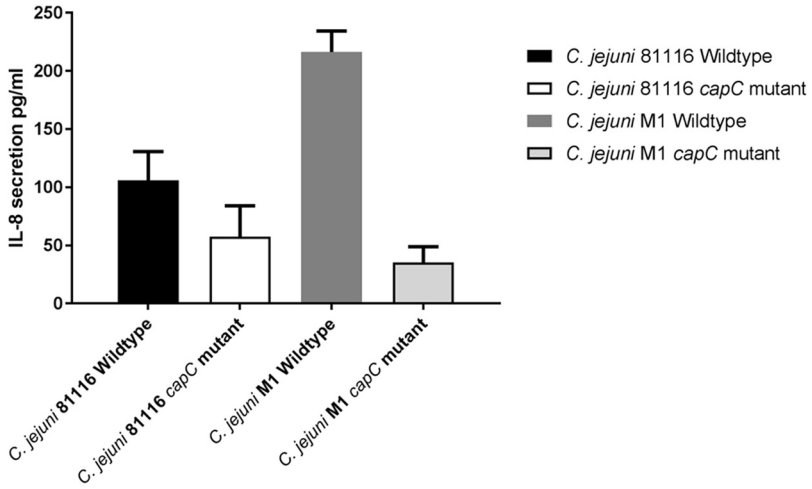

FIG 6 Decrease in IL-8 secretion from T84 cells in response to infection with wild-type *C. jejuni* and *capC* mutant strains (MOI of 1:100). IL-8 secretion is shown in picograms per milliliter. Infection of T84 cells with *capC* mutants caused a decrease in the levels of IL-8 secretion from T84 cells. The greatest decrease was observed in *C. jejuni* M1 *capC* ($P = 0.0081$). Error bars show standard errors of the means. This assay was performed in quadruplicate on three occasions.

consistent with previously characterized autotransporter proteins, including the N-terminal signal sequence and the C-terminal barrel domain (30). We have shown that CapC is an outer membrane protein as predicted using bioinformatic tools; this is another feature that is consistent with CapC belonging to the autotransporter family. Given the functions of autotransporters, CapC is a putative virulence determinant in *C. jejuni* that may contribute to infection processes.

The larvae of the greater wax moth (*Galleria mellonella*) have been used previously as an effective and powerful model to identify putative virulence determinants in a range of pathogens, including *C. jejuni* (49–52). This model has been used to demonstrate that mutants with deletions in factors that have previously been implicated as virulence determinants elicit decreased mortality in the *Galleria* model, relative to wild-type strains (45, 53). In addition to being an effective and ethical alternative to animal models, the *Galleria mellonella* virulence model provides a quantifiable measure of virulence within the context of a functional immune system (52, 54, 55).

We have demonstrated that inactivation of *capC* impairs virulence of *C. jejuni* in *Galleria* larvae as determined by the mortality/survival rates and severity of disease (morbidity). However, the mechanism by which CapC contributes to insecticidal activity and therefore virulence in this model is unclear. For example, loss of capsule modification capability conferred by *O*-methyl phosphoramidate (MeOPN) of *Campylobacter* has been shown to cause reduced virulence in the *Galleria* model (45, 56). MeOPN has been shown not to have inherent insecticidal activity however (45), suggesting that reduced virulence is a result of impaired bacterial fitness. Conversely, inactivation of the Dot/Icm type IV secretion system in *Legionella pneumophila* abolished virulence in *Galleria*, suggesting that intracellular survival and bacterial proliferation are key to virulence (57). Given that the absence of CapC results in decreased adhesion to human intestinal epithelial cells, it is likely that reduced virulence associated with *capC* mutants is due to reduced interaction with epithelial cells. *Galleria mellonella* and other insects, such as *Drosophila*, exhibit a melanization response upon infection (49). The melanization process is an innate immune response mediated by phenoloxidase (PO) (49, 52, 58). Melanization is triggered by recognition of microbial products, which are often surface exposed, by soluble pattern recognition receptors. This causes a serine protease cascade resulting in cleavage of prophenoloxidase (ProPO) to PO, which in turn, converts phenols to quinines that polymerize to form melanin (48, 50). Melanin is deposited to encapsulate pathogens, resulting in pigmentation of the larvae (49, 58).

This process is analogous to abscess formation induced by mammalian infections (49) and can be considered to be part of the inflammatory immune responses of these insects.

In this study, we have demonstrated that the absence of CapC results in a reduced immune response from *Galleria,* as wild-type strains consistently caused a significantly greater degree of color change in *Galleria*, indicative of a greater degree of melanization. The absence of CapC also resulted in a reduced inflammatory response from human intestinal cells, as wild-type strains elicited higher IL-8 secretion levels than *capC* mutants did. IL-8 is a proinflammatory cytokine that plays a key role in mobilizing cellular defense mechanisms. *C. jejuni* has been demonstrated to stimulate IL-8 secretion from human intestinal cells *in vitro* via the NF-κB pathway, which is a major downstream target of Toll-like receptor (TLR) signaling pathways (59). Therefore, any change in IL-8 secretion associated with inactivation of *capC* may be indicative of a change in interaction of *C. jejuni* with the epithelial cell surface. Furthermore, it has been shown that *C. jejuni*-induced IL-8 secretion via mitogen-activated protein (MAP) kinase stimulation is dependent upon gene products that are expressed upon contact with epithelial cells (59, 60). The degree of IL-8 secretion in *C. jejuni* M1 was considerably greater than that elicited by *C. jejuni* 81116 which correlates with the virulence profiles of these strains in the *Galleria* model (Fig. 6). Despite causing high levels of morbidity and mortality at 24 h postinfection, the comparatively low IL-8 secretion elicited by wild-type *C. jejuni* 81116 at 4 h postinfection is likely due to the lower growth rate of this strain (Fig. S4). Previous studies have shown that the flagella, cytolethal distending toxin (CDT), and outer membrane vesicles (OMVs) of *Campylobacter* have been shown to elicit secretion of IL-8 via activation of TLRs (59). Whether CapC elicits secretion of IL-8 directly or merely inhibits the capacity of other factors to do so is unclear; however, the observed decrease in IL-8 secretion suggests that the absence of CapC affects interactions with epithelial cells.

In addition to fibronectin binding proteins such as CadF and FlpA, factors such as JlpA (61), Peb1a (62), and CapA (22) are reported to influence adhesion to different degrees. This multitude of reported adhesins has complicated understanding of the adhesion process, and is further complicated by the failure to distinguish between factors contributing to bacterial fitness and "true" adhesins involved in direct interaction with the host (62). Furthermore, most of the reported adherence-impaired single gene mutants with mutations in genes encoding the aforementioned factors exhibit only reduced rather than abolished interactions with eukaryotic cells (63). This observation suggests that redundancy of factors may exist in the adhesion process, a compelling notion considering the number of reported factors, and the fact that certain adhesins are not conserved among *C. jejuni* lineages (22). A combination of nonpolarized, partially differentiated Caco-2 cells and nonpolarized T84 cells were used to demonstrate that CapC contributes to adhesion in these models. Adhesion and invasion efficiencies of *capC* mutants were decreased in the T84 cell model and significantly decreased in the Caco-2 cell model. Subsequent comparison of the levels of invaded *C. jejuni* as a proportion of associated bacteria revealed that mutant strains showed marginally increased proportional invasion. Therefore, the observed phenotypes indicate that CapC is required for maximal adhesion to human intestinal cells. Identification of CapC as an additional autotransporter contributing to adhesion suggests that *C. jejuni* does not have a defined, uniform method of adhesion and invasion of intestinal cells. The results presented here support a case that adhesion, and consequently invasion, are dependent on the cooperative action of several different factors. Moreover, certain adhesins are not conserved between different *C. jejuni* strains, which indicates that different *Campylobacter* lineages have different mechanisms of interaction with the host and different strategies of colonization.

Identification of a specific role for CapC in the human niche raises the question as to whether CapC plays a role in the interaction with the chicken host. The outer membrane is the interface for interaction with the host and environment, and many genes involved in interaction with the human host are also implicated in colonization

of the chicken (6, 42, 64, 65). For example, CadF and FlpA bind fibronectin in both chicken and human cell models, although they do differ in their expression in each host (5, 14). The CapA autotransporter is involved in adherence to both human intestinal epithelial cells and chicken liver epithelial cells and has been linked to chicken colonization, although it has also shown to be dispensable in certain strains (5, 22). Additionally, a *cj0511* serine peptidase mutant that was attenuated in *G. mellonella*, was shown to have severely reduced capacity for chicken colonization (66).

In this study, we have investigated the contribution of CapC to the virulence of *Campylobacter*. We have shown that CapC possesses a number of features consistent with autotransporters, including the autotransporter domain which is unique to members of this protein class. Using the *Galleria mellonella* model, we have shown that *capC* significantly contributes to virulence *in vivo*, and likely functions as an adhesin and immunogenic factor. Furthermore, our findings highlight that *Campylobacter* uses a variety of mechanisms involved in virulence, with many of these being multifactorial and variable between isolates and genetic backgrounds.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Campylobacter jejuni* 81116 (NCTC 11828) and *C. jejuni* M1 and their respective *capC* mutant derivatives were routinely cultured on Mueller-Hinton agar (MHA) supplemented with 5% sheep blood or in Mueller-Hinton broth (MHB) and incubated at 42°C in a microaerobic atmosphere generated utilizing Campygen gas packs (Oxoid, UK) (5% O_2, 10% CO_2, and 85% N_2). Cultures were supplemented with trimethoprim (100 μg ml^{-1}), chloramphenicol (10 μg ml^{-1}), and/or kanamycin (50 μg ml^{-1}) where appropriate. *Escherichia coli* DH5α and *E. coli* JM109 were used for propagation of plasmids, and constructs were maintained in Luria-Bertani broth supplemented with 25% glycerol at −80°C in 1.5-ml vials. *E. coli* strains were cultivated in an aerobic environment at 37°C for 16 to 24 h.

Bioinformatic tools and sequence analysis. The nucleotide sequence and predicted protein sequence encoded by *capC* were downloaded in Fasta format from the CampyDB database resource at http://xbase.warwick.ac.uk/campydb/. CapC is annotated as C8J_1278 in *C. jejuni* 81116 and CJM1_1321 in *C. jejuni* M1. These sequences were used for all bioinformatic analyses. The NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) is a publically available tool that can be used to annotate conserved domains in protein sequences. This tool was used to identify potential conserved domains in *capC*. The SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict putative signal peptides within CapC. The subcellular localization prediction tools Cello V2.5 (http://cello.life.nctu.edu.tw/) and PSORTb (http://www.psort.org/psortb/index.html) were used to predict the localization site of CapC. Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to build a partial three-dimensional (3D) model of the CapC protein structure based on remote homology detection methods.

Generation of *capC*-deficient mutants. The *capC* gene in *C. jejuni* strains 81116 and M1 was inactivated using a chloramphenicol resistance cassette insert. The *capC* gene was amplified from *C. jejuni* strains 81116 and M1 using the CapCFW (FW stands for forward) and CapCRV (RV stands for reverse) primers (Table 1), resulting in a 2,893-bp fragment. This fragment was cloned into pGEM-T Easy, and the resulting pJM005 was digested with BglII and XbaI, cleaving the *capC* sequence after nucleotide positions 1501 and 2164, respectively, and thus excising a 647-bp *capC* fragment from the plasmid. The chloramphenicol resistance (*cat*) cassette was amplified from pAV103 using the primers JMCAT5 and JMCAT6 (67), digested with BglII and XbaI, and ligated into pJM005, thereby creating pJM007. A 1,906-bp fragment amplified from pJM007 using the Vac1 and Cap3 primers was used to transform *C. jejuni* 81116 and M1 by electroporation by the method of Holt et al. (68). Table 1 and Table S2 in the supplemental material detail all oligonucleotide primers and plasmids used in this study, respectively.

Isolation of cellular fractions. *C. jejuni* was harvested from agar plates and diluted in 10 mM HEPES (Sigma, UK) to an optical density at 600 nm (OD_{600}) of 0.8 (approximately 5×10^7 CFU/ml). These bacterial suspensions were used to isolate supernatant, outer membrane, inner membrane, and cytoplasmic fractions by the method of Sommerlad and Hendrixson (69).

Antiserum generation and immunoblot analysis. Rabbit polyclonal antibodies specific to CapC were generated using a synthetic peptide (EKLQQEAMQNGGKIDDEKY) designed with the Antigen Profiler software provided by Life Technologies (Thermo Fisher). New Zealand White rabbits were immunized over a period of 70 days and bled three times to obtain crude antibody sera. Immunoblotting was performed as described previously with minor modifications (69).

Membrane fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12.5% Novex Tris-glycine precast protein gels (Thermo Scientific, UK), and proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Fisher, UK) using a Pierce Power Blot cassette (Thermo Scientific, UK) using Pierce one-step transfer buffer (Thermo Scientific, UK). PVDF membranes were probed with primary anti-CapC antibody diluted 1:1,000 in 10 ml Tris-buffered saline with Tween 20 (TBST) and 3% milk powder for 1 h at room temperature. The membranes were washed and probed with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (catalog no.

TABLE 1 Oligonucleotide primers used in this study

Primer name[a]	Primer sequence (5′–3′)[b]	Restriction site[c]	Reference
Cap1	CAATTAGGAAGTATAGGCGCA	N/A	This study
Cap2	AGCACCCACTTCAACACTTAC	N/A	This study
Vac1	GTGTAATCATCACCACTATTGATA	N/A	This study
JMCAT5	AAAAGATCTCTCGGCGGTGTTCCTTTCCAAG	BglII	This study
JMCAT6	GATTCTAGACGCCCTTTAGTTCCTAAAGGGT	XbaI	This study
CC069	ATATGTGCAGGGCGTATTGC	N/A	37
Cj0046F	CACTACCCAATTGAAAATCTAAG	N/A	72
Cj0046R	GTGAGTTAATGCCATCATAAC	N/A	72
Cap3	CCAAATGGATTATCAAGGC	N/A	This study
Cap4	GCTTGTTTATCTACTCCCACGG	N/A	This study
CapC-Fw	ACTTGGCTTCGACAGGAGT	N/A	This study
CapC-Rv	AAGATAAAGCCCTAGGAGATTTC	N/A	This study
BamCapC-FW	GATGGATCCTGAAGGAGAAACTTATGAAG	BamHI	This study
BamCapC-RV	GATGGATCCAAGATAAAGCCCTAGGAGATTTC	BamHI	This study
pSV009FW1	TAATAGAAATTTCCCCAAGTCCCA	N/A	37
pSV009RV1	CTATTGCCATAGTAGCTCTTAGTGG	N/A	37
CapCFBam	GATGGATCCACTTGGCTTCGACAGGAGT	BamHI	This study
CapCRXho	GATCTCGAGAAGATAAAGCCCTAGGAGATTTC	XhoI	This study

[a]Forward primers are indicated by F, Fw, or FW at the end or in the middle of the primer designation, and reverse primers are indicated by R, Rv, or RV at the end or in the middle of the primer designation.
[b]The restriction sites are shown underlined.
[c]N/A, not applicable.

ab6721; Abcam) diluted 1:5,000. Exposure and image capture were performed using a Licor Odyssey Fc imager.

***Galleria mellonella* virulence model.** *Galleria mellonella* larvae were purchased from commercial suppliers Livefood or Livefoods Direct. Use of this model provides the basis for a rapid screening of virulence phenotypes within the context of a functional immune system comparable to that of mammals. Factors implicated as integral to *Campylobacter* infection of the human host have also been shown to contribute to insecticidal activity in this model (51, 66, 70). Larvae were shipped at the fifth- or sixth-instar stage (approximately 2 to 3 cm in length) and were suitable for use immediately. Larvae were maintained at 17°C on wood chips for up to 2 weeks. All larvae were handled using ethanol-sterilized blunt-nosed forceps and examined prior to infection; any larvae showing discoloration or signs of pupation were immediately discarded and euthanized. Healthy larvae were selected on the basis of their uniform creamy color with no areas of dark discoloration and that they were able to right themselves quickly if turned over. *C. jejuni* strains were grown overnight and resuspended in phosphate-buffered saline (PBS) to an OD_{590} of 1 (1×10^8 CFU/ml). This bacterial suspension was used to infect *Galleria* larvae. Larvae were injected into the top right proleg with 10 μl bacterial cell suspension. Injection was performed using a Hamilton 26S Microliter syringe (Sigma). Once infected, larvae were placed on a Whatman 90-mm filter paper in an inverted petri dish and incubated at 37°C for up to 48 h. A negative control in which larvae were injected with PBS only and a noninfected control were also included. Ten larvae were infected for each isolate in triplicate on four separate occasions. Mortality was confirmed by the inability of larvae to respond to physical stimuli (56).

Cell lines, media, and culture conditions. Caco-2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids and 1% (wt/vol) 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. T84 cells were maintained in DMEM/F-12 Ham's nutrient mix (Invitrogen) supplemented with 10% (vol/vol) fetal calf serum (FCS), 1% (wt/vol) nonessential amino acids, and 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. For certain assays, the medium from Caco-2 or T84 cells was removed, and the monolayers were washed twice with PBS and then maintained in antibiotic-free and/or serum-free DMEM/F-12 Ham's nutrient mix.

Adhesion and invasion assays. CFU-based adherence assay and gentamicin protection assays were conducted as described previously with minor modifications (70, 71). Caco-2 cells were seeded for 1 week in 24-well plates at 1×10^6 cells ml^{-1} in 1 ml of DMEM in 24-well plates and infected with 100 μl of *C. jejuni* suspensions prepared as described above at a multiplicity of infection (MOI) of 1:200. The cells were incubated at 37°C for 3.5 h in a 5% CO_2 humidified incubator. Monolayers were lysed with prewarmed 1% (wt/vol) Triton X-100 to release cell-associated bacteria which were enumerated. A gentamicin protection assay was performed in conjunction with the adhesion assay. After incubation for 3.5 h, the medium was removed, monolayers were washed, and complete DMEM supplemented with gentamicin to a final concentration of 150 μg ml^{-1} was added to each well. The plates were reincubated for 2 h at 37°C followed by bacterial enumeration.

Adhesion and invasion efficiencies were calculated as described previously (71). Additionally, the number of invaded bacteria as a proportion of associated bacteria (the number counted in adherence assays) was calculated.

Cytotoxicity assay. Cytotoxicity assays were performed using the Pierce lactate dehydrogenase (LDH) cytotoxicity assay kit according to the manufacturers' instructions (Thermo Fisher, UK). Caco-2 cells were seeded at 1×10^5 per well of a 96-well plate in 100 μl of complete DMEM for 48 h. Cultures of

wild-type *C. jejuni* and *capC* mutants grown in broth for 24 h were resuspended in PBS to an OD_{590} of 1.0, with 10 μl of this suspension being used to inoculate the appropriate wells in triplicate. Serum-free DMEM and complete DMEM without cells were included to account for LDH activity in sera. The plates were incubated for 4 h at 37°C in a 5% CO_2 humidified incubator. Absorbance was measured at 490 nm and 680 nm using a Wallac plate reader, and the percentage of cytotoxicity was calculated. These assays were performed in quintuplet on three different occasions.

ELISA for IL-8. The levels of IL-8 secretion from T84 cells were assessed using the eBioscience IL-8 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. T84 cells were infected with *C. jejuni* as described above. After 4-h incubation, the infected-cell supernatant was removed and added to the wells of a 96-well Nunc Maxisorp plate, in quadruplicate, which had been preblocked and exposed to a capture antibody specific for IL-8. The plates were incubated overnight, and the levels of IL-8 secretion were determined according to the manufacturer's instructions. OD was measured on a Wallac plate reader at 450 nm, and the levels of IL-8 secretion were determined. This assay was repeated three times.

Statistical analysis. All statistical analyses were performed with Graphpad Prism (v 7.0; Graphpad Software). Data from wild-type and *capC* mutants were compared using a Mann-Whitney test or an unpaired *t* test. Differences were considered significant at a P of <0.05. Virulence in the *Galleria mellonella* model was compared using multiple *t* test function in Graphpad Prism v 7.0 (false-discovery rate of 0.1) to obtain adjusted P values (q values).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01032-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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