

Campylobacter capsule and lipooligosaccharide confer resistance to serum and cationic antimicrobials

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Key words: campylobacter, innate immunity, host defense, lipooligosaccharide, capsule, complementation, serum, cationic antimicrobial

Abbreviations: BPI, the bactericidal/permeability-increasing protein; CAPs, cationic antimicrobial peptides and proteins; cfu, colony-forming units; Crp4, cryptdin-4; HINHS, heat-inactivated normal human serum; hNGP, human neutrophil granule protein extract; LPS, lipopolysaccharide; LOS, lipooligosaccharide; MIC, minimal inhibitory concentration; NHS, normal human serum; TSA, trypticase soy agar; rBPI-21, twenty-one kilodalton fragment of recombinant BPI

The innate immune system plays a critical role in host defense against mucosal bacteria. *Campylobacter jejuni* is a major cause of human gastroenteritis that usually resolves spontaneously within several days, suggesting that innate mechanisms are important to control the infection. However, the specific means by which this occurs is not well understood. While diarrheal isolates of *C. jejuni* usually are susceptible to human serum, we found that a systemic strain of *C. jejuni*, isolated from the cerebrospinal fluid of an infant with meningitis, is relatively more resistant to human serum, the Bactericidal/Permeability-Increasing Protein (BPI), an endogenous cationic antimicrobial protein, and the cationic peptide antibiotic polymyxin B. To test the hypothesis that the surface properties of this strain contributed to its ability to withstand these innate host defenses, we constructed isogenic mutants in capsule (*kpsM*) and lipooligosaccharide (*waaF*) and complemented these mutants by insertion of the complementation construct in trans into *hipO*, a chromosomal locus. We found that capsule expression was essential for serum resistance, whereas lipooligosaccharide played no substantial role. In contrast, the lipooligosaccharide mutant showed increased sensitivity to polymyxin B, α -defensins, cathelicidins and BPI. These findings suggest that the polysaccharides of *C. jejuni* strains contribute differently to resistance against host innate immunity, whereby capsule is more important for resisting human complement and lipooligosaccharide is more important for protection against killing mediated by cationic antimicrobial peptides and proteins.

Introduction

Campylobacter jejuni is a microaerophilic Gram-negative rod that is a common cause of food-borne diarrheal illness in humans.^{1,2} This self-limited disease is characterized by fever, abdominal pain and bloody diarrhea that usually resolves within several days of onset of symptoms,² suggesting that host innate defenses contribute to the resolution of infection.^{3,4} Although the illness is often mild, *C. jejuni* infections occasionally lead to complications such as bacteremia, post-infectious reactive arthritis or Guillain-Barré Syndrome.^{2,5,6} Despite the observation that these bacteria are able to persistently colonize a variety of livestock, particularly fowl,⁷ relatively little is known about the mechanisms they use to evade host innate immune mechanisms.

Numerous studies have examined the role of bacterial surface structures, protein glycosylation, toxins and the invasion of

intestinal epithelial cells that are important for *C. jejuni* diarrheal pathogenesis.⁸⁻¹¹ However, less is known about the pathogenesis of *C. jejuni* infections that gain access to the vascular space and cause systemic infection. When a systemic *C. jejuni* infection occurs in an apparently immunocompetent individual, the recovered isolate is likely to have features promoting persistence in the bloodstream that distinguish it from diarrheal *C. jejuni* strains.¹²

The vertebrate intestinal tract relies on both innate and adaptive immune defenses to limit both the residential and the acquired, often pathogenic, microbiota from traversing the intestinal mucosa and entering the host bloodstream.^{13,14} Examples of important components of the innate immune system with direct bactericidal activity include cationic antimicrobial peptides and proteins (CAPs) and complement.^{13,15-17} For example, in the intestine, the inducible secretion of one family of CAPs known as defensins contributes to innate defenses.^{15,18} A second

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Submitted: 08/27/10; Revised: 01/05/11; Accepted: 01/10/11
DOI: 10.4161/viru.2.1.14752

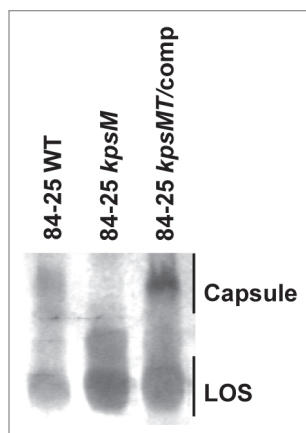


Figure 1. Analysis of capsule and LOS expression in a systemic *C. jejuni* strain. Whole cell proteinase K-digested lysates of wild-type *C. jejuni* 84-25 (84-25 WT), capsule mutant (84-25 *kpsM*), and the complement (84-25 *kpsMT/comp*) were separated on 12% SDS-PAGE and stained with 0.5% Alcian Blue to reveal capsular polysaccharides and with silver to stain LOS.

family of CAPs, the cathelicidins, is produced principally by neutrophils and effect host defense both in the vascular space and in tissue sites of infection after neutrophil recruitment.^{19,20} Another cationic neutrophil-derived antimicrobial is the 55 kDa Bactericidal/Permeability-Increasing Protein (BPI), which is stored in the primary granules of neutrophils and shows potent and selective antimicrobial activity against Gram-negative bacteria via its affinity for the lipid A portion of lipopolysaccharide (LPS) and lipooligosaccharide (LOS).^{17,21} The importance of CAPS in host defense against *C. jejuni* is supported by the observation that the diarrheal strain 11168 stimulates upregulation of human β -defensins in intestinal epithelial cells in vitro and is susceptible to their activity.⁴

C. jejuni has a highly expressed and varied surface glycome, and many glycosylated structures are associated with surface components including the capsule polysaccharide and the oligosaccharide side-chains of its LOS.²² Such surface structures are in direct contact with components of innate host immunity, and so likely play biologically important roles in *C. jejuni*. The importance of complement in defense against bloodstream pathogens in general²³ and diarrheal *C. jejuni* strains specifically are well-known,^{2,24} and has been attributed to both capsule and LOS in these diarrheal isolates.^{24,25} Specifically, truncation of sialic acid from the LOS cores of certain diarrheal strains lead to increased sensitivity to CAPs such as the cathelicidin LL-37 and polymyxin B.²⁴ In the current work, we explore how the surface structures of systemic *C. jejuni* isolated affect susceptibility to complement and CAPs.

To achieve this, we focused on an invasive *C. jejuni* strain 84-25, which was isolated from the cerebrospinal fluid of an apparently immunologically normal child with meningitis.¹² We also examined strain 84-19, another *C. jejuni* isolate from the cerebrospinal fluid of an infant with meningitis, with the expectation that both strains have similar surface characteristics. Although antecedent bacteremia was not documented in these

children with *Campylobacter* meningitis, hematogenous spread to the meninges from the gut is the most likely sequence of events.

We hypothesized that capsule and LOS from strain 84-25 contribute to its virulence via resistance against serum and CAPs. By construction of isogenic acapsular and LOS truncation mutants of this systemic isolate, we assessed the contribution of its glycome to evasion of the antimicrobial properties of serum and cationic antimicrobial effectors. To complement these mutants, we developed a chromosomal complementation method. We used the *hipO* locus, encoding the non-essential enzyme hippurate hydrolase, which degrades hippuric acid into benzoic acid and glycine.²⁶ Since this enzyme is highly conserved in *Campylobacter*,²⁷ this complementation strategy can be used in nearly all *C. jejuni* strains and so represents a potential advance over previously published methods.^{8,11,28-30}

Results

Characterization of capsule expression in the *C. jejuni kpsM* mutants. Capsule produced by the wild-type strain 84-25, its capsule mutant (*kpsM*), and in trans complemented mutant (*kpsMT/comp*) were characterized by SDS-PAGE, followed by Alcian Blue staining (Fig. 1). As expected, the *kpsM* mutant showed decreased capsule expression compared to the wild-type strain. A Shine-Dalgarno sequence is present upstream of *kpsM* but not *kpsT*, suggesting translational coupling;³¹ therefore, complementation with *kpsM* alone did not restore capsule expression (data not shown) whereas complementation with *kpsMT* did, as noted by others in strain 81-176²⁵ (Fig. 1). We also examined strain 84-19, another *C. jejuni* isolate from the cerebrospinal fluid of an infant with meningitis. Although the capsules of strains 84-25 and 84-19 display nonidentical electrophoretic mobility (data not shown) and are Penner serotype 2 and 13, respectively,¹² similar results were observed in strain 84-19 and its isogenic *kpsM* mutant (data not shown). These results demonstrate that *kpsM* participates in capsule assembly of strain 84-25, as has been shown in other *C. jejuni* strain backgrounds.³¹

In silico analysis of LOS biosynthetic loci. To study the contribution of the LOS core structures of diarrheal and systemic strains to resistance to host defenses, we first performed an in silico analysis of the LOS biosynthetic loci. We found that strain 84-25 and NCTC 11168 share greater than 98% average identity among homologous glycosyltransferases and sialic acid synthesis genes (Fig. 2). Although *orf1164* of strain 84-25 is present on the minus strand, there was 100% identity between CJ1137 (strain 11168) and *orf11644*, suggesting they are orthologs. Therefore, strain 84-25 possesses a class C LOS locus,^{32,33} and likely expresses an inner-core LOS that is nearly identical to the diarrheal strain 11168.³⁴ The genomic features that distinguish strains 84-25 and 81-176 include the presence of *galT* in 81-176 and the presence of *wlaN* in strain 84-25. We noted that *orf1156* in strain 81-176 showed partial identity to the 5' region of *wlaN*, however there was no corresponding 3' region present for it to be considered an ortholog by ClustalX alignment. However, both *galT* and *wlaN* attach carbohydrate moieties to the outer-core region of LOS, whereas the inner LOS cores of the diarrheal strains and the

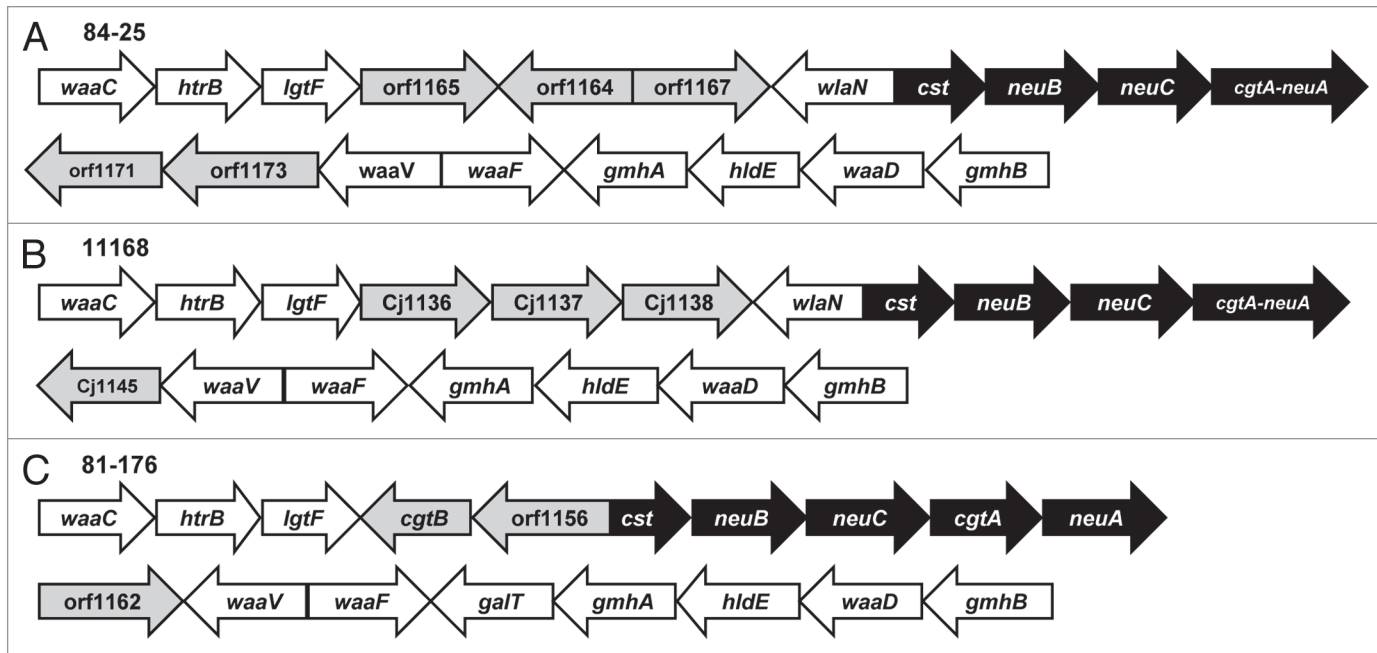


Figure 2. Schematic diagram of the LOS biosynthetic loci. *C. jejuni* strain, 84-25 (A) and 11168⁶⁸ (B), and 81-176⁶⁹ (C), were analyzed in silico to determine homology within the LOS biosynthetic loci. The nomenclature reflects the open reading frame annotations of the respective strains, except where homology has been noted in the NCBI records (i.e., as in *waaC* and *hldE*). Pair-wise alignment revealed average identity of 98–99% among homologous glycosyltransferase (white arrows) and sialic acid synthesis genes (black arrows). Hypothetical proteins are shaded in grey. In strains 84-25 and 11168, the *cgta* and *neuA* genes occur as an in-frame fusion ORF.^{9,32,70}

systemic strain 84-25 are likely to be nearly identical, based on published structures.^{9,34}

Characterization of the LOS of the *C. jejuni* 84-25 *waaF* and 84-19 *waaF* mutants. The LOS molecules produced by wild-type strains 84-25 and 84-19 and their *waaF* mutants were characterized by Tricine-PAGE. As expected,³⁵ silver staining revealed that the LOS produced by the *waaF* mutant strains was truncated compared to that produced by their respective wild-type cells (Fig. 3). Complementation of the 84-25 mutant restored expression of full-length LOS. The results demonstrate that disruption of *waaF* in *C. jejuni* 84-25 and 84-19 leads to expression of a truncated LOS, which can be restored to full-length in strain 84-25 by complementation of *waaF* in the *hipO* locus.

Effect of LOS truncation and capsule loss on resistance to antimicrobial agents. To cause illness, enteric bacteria must survive exposure to host mucosal innate defenses. CAPs play a major role in defending mucosal surfaces from pathogens,³⁶ some of which have developed strategies to overcome these innate defenses such as encapsulation and/or production of full-length LPS or LOS.^{15,16,36,37} To test the hypothesis that such bacterial phenotypes would contribute to *Campylobacter* resistance to CAPs, we first tested the antimicrobial property of polymyxin B, a cyclic, lipid A-interactive peptide antibiotic because it is a model compound for assessing resistance to CAPs.³⁸ Using an agar-based assay, the systemic *C. jejuni* strains 84-19 and 84-25 were two- to ten-fold more resistant to polymyxin B compared to diarrheal strains 81-176 and 79-193 (Table 3). Of the systemic strains, their corresponding LOS-truncation mutants showed four- to six-fold increased sensitivity to polymyxin B, as indicated

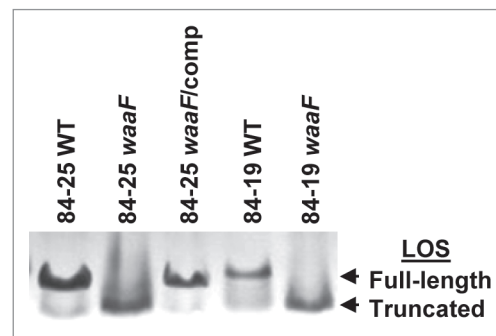


Figure 3. Analysis of LOS expression in *C. jejuni* strains. Bacterial lysates of the strains in Figure 1 and wild-type *C. jejuni* 84-19 (84-19 WT) and the LOS mutant (84-19 *waaF*) were resolved by 15% Tricine SDS-PAGE and silver-stained to reveal LOS.

by their lower MICs ($p < 0.005$). These data are consistent with findings by others using the diarrheal strain 81-176 and LOS truncation mutants.²⁴ Complementation of *waaF* in strain 84-25 restored most polymyxin B resistance. Disruption of capsule or LOS expression did not affect susceptibility of mutants to streptomycin or nalidixic acid, which kill by inhibition of protein synthesis and DNA replication, respectively. Furthermore, polymyxin B sensitivity was unchanged in the capsule mutant of 84-25 compared to the wild-type strain, suggesting that this surface constituent does not play a major role in polymyxin B resistance.

Effect of LOS truncation on susceptibility to host CAPs. Given our finding that full-length LOS confers resistance against

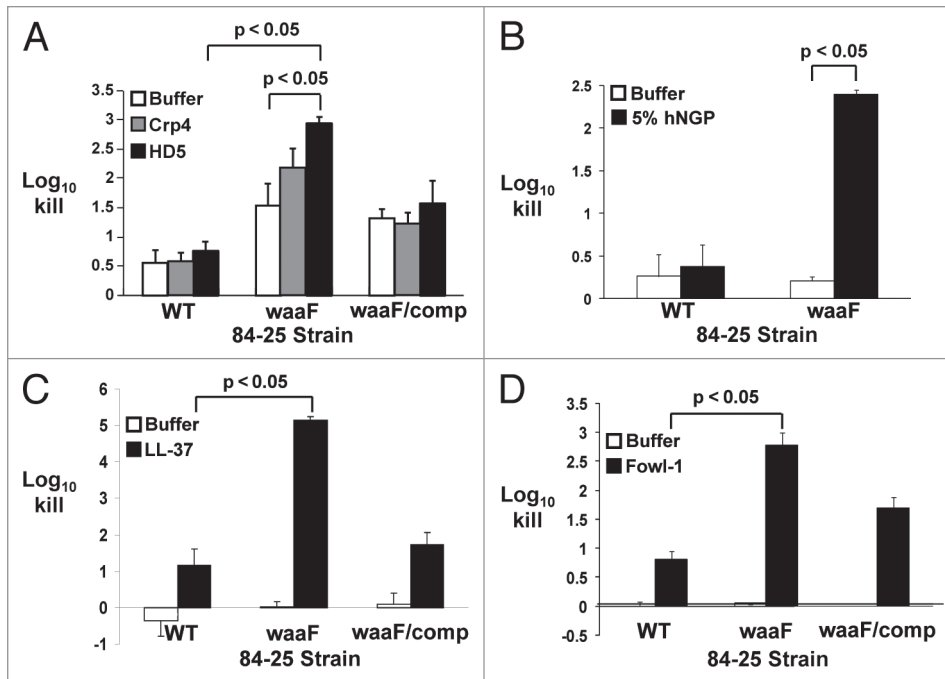


Figure 4. Killing of *C. jejuni* strains by human neutrophil granule extract and cationic antimicrobial peptides. The wild-type strain of *C. jejuni* 84-25 (WT), the LOS mutant (*waaF*) or the complement (*waaF/comp*) were incubated with cationic antimicrobial peptides (HD-5, Crp4, LL-37, Fowl-1; black bars), human neutrophil granule protein extract (hNGP; black bars) or buffer (white bars). Data are expressed as the \log_{10} kill. (A) Wild-type *C. jejuni* strain 84-25 (WT), the LOS mutant (*waaF*) or the complement (*waaF/comp*) were incubated with the murine α -defensin homolog cryptdin-4 (Crp4), human α -defensin-5 (HD-5) (both 10 $\mu\text{g}/\text{ml}$) or buffer x 120 min. (B) *C. jejuni* 84-25 (WT) or the LOS mutant (*waaF*) were incubated with 5% hNGP or buffer x 30 min. (C) The strains in (A) were incubated with the human cathelicidin LL-37 (10 $\mu\text{g}/\text{ml}$) or buffer x 30 min. (D) The strains in (A) were incubated with the chicken cathelicidin homolog fowlicidin-1 (10 $\mu\text{g}/\text{ml}$; fowl-1) or buffer x 60 min. Data are the mean \pm SD of at least two replicates performed 3–7 times. Significance ($p < 0.05$) was determined by student's two-tailed t-test.

polymyxin B-mediated bacterial killing (Table 3), we hypothesized that LOS would similarly provide protection against CAP-dependent killing. Alpha defensins are a class of CAPs specifically secreted by Paneth cells into the lumen of the small intestine in response to bacterial surface structures,³⁹ and likely would be the first and most potent intestine-derived CAPs to engage bile-resistant *C. jejuni*. Whereas wild-type *C. jejuni* 84-25 was relatively resistant to human α -defensin-5 and its murine homolog Crp4,⁴⁰ the LOS mutant showed 2–3 \log_{10} increased killing by Crp4 and human α -defensin-5 (Fig. 4A; $p < 0.05$ for the latter compared to buffer alone). As with polymyxin B, these data are consistent with findings by others using the diarrheal strain 81-176 and LOS truncation mutants.²⁴ Complementation restored survival to near-wild-type levels. These data indicate that the distal LOS afforded protection against these CAPs.

Effect of LOS truncation on susceptibility to neutrophil-derived CAPs. Bacteria that evade intestinal luminal defenses may next invade the mucosa and gain systemic access, where they are exposed to additional cellular and humoral elements of the innate immune system. A key component of the cellular arm of innate immunity includes neutrophils in humans and heterophils in chickens. Neutrophils in humans are the major source of cathelicidins such as LL-37,⁴¹ and in chickens, the heterophil-derived

cathelicidin homolog fowlicidin-1.⁴² To counteract these host defenses, some microbes have evolved surface-dependent virulence strategies; for example, in *Neisseria meningitidis*, LOS limits the access of the CAP LL-37 to susceptible hydrophobic membranes and mitigates killing compared to an LOS-deficient strain.⁴³ Therefore, as a first step towards determining whether LOS plays a similar role in *C. jejuni*, we assessed the susceptibility of wild-type and LOS-truncated *C. jejuni* 84-25 measured the antimicrobial activity of human neutrophil granule protein (hNGP) extract, a complex mixture that contains, in addition to defensins, the majority of the host's cathelicidins and BPI.^{15,21,44} We found that the 84-25 *waaF* mutant was >100-fold more sensitive than the wild-type cells to hNGP ($p < 0.05$) (Fig. 4B). Based on this finding, we next determined whether the distal LOS protected *C. jejuni* from specific CAPs from humans and other species. The human CAP cathelicidin LL-37 caused >10-fold increased killing of the LOS-truncated strain of 84-25 versus the wild-type strain (Fig. 4C). Similarly, the LOS mutant showed ~3 \log_{10} -fold increased susceptibility

to the chicken cathelicidin homolog fowlicidin-1 ($p < 0.05$) compared to the wild-type strain (Fig. 4D). Complementation of the *waaF* gene conferred near-complete restoration of resistance to these cathelicidins ($p < 0.05$) (*waaF/comp*, Fig. 4C and D). Thus, similar to findings for the diarrheal strain 81-176 shown by others,²⁴ full-length LOS provided considerable protection against the CAP constituents of innate immunity for these systemic *C. jejuni* isolates.

Susceptibility of diarrheal and systemic strains to neutrophil-derived rBPI-21 and effect of LOS truncation. In addition to defensins and cathelicidins, neutrophils are the major source of the cationic protein BPI, which, like other CAPs, exerts selective activity against Gram-negative bacteria via its binding affinity for the lipid A portion of LPS/LOS.¹⁷ Since all of the antimicrobial activity of BPI is contained within its N-terminal half,⁴⁵ we used this 21 kDa fragment in recombinant form (rBPI-21) to compare the susceptibility of a diarrheal strain (81-176) and our systemic strain 84-25 to BPI-mediated killing. Our positive control for BPI-21 antimicrobial activity was *E. coli* strain K1/r, known to be susceptible to BPI-mediated killing⁴⁶ and which showed 1–4 \log_{10} killing under these conditions (Fig. 5B). When we compared killing of the *C. jejuni* diarrheal and systemic strains at the highest BPI dose, we found 0.4 \log_{10} increased killing of the

diarrheal strain ($p < 0.0001$); the difference also was significant at the 30 nM dose (Fig. 5A). To measure the contribution of the distal LOS to the resistance of strain 84-25 to BPI-mediated killing, we next measured the effect of LOS truncation. We found dose-dependent, 2–3 \log_{10} increased killing of the LOS mutant compared to the wild-type strain ($p < 0.005$), a phenotype partially restored by complementation of *waaF* (Fig. 5B). These data are consistent with our data showing increased sensitivity of the LOS mutant to human neutrophil granule protein extract and CAPs (Fig. 4). Overall, these results are consistent with studies in the *E. coli* showing that LPS chain length determines susceptibility to BPI-dependent killing,^{47,48} and indicate that the distal LOS of strain 84-25 contributes to protection against both luminal and systemic CAPs.

Effect of LOS truncation and capsule loss on complement-mediated bacterial killing by serum. The bactericidal activity of complement present in serum is an important innate defense against bacteria that gain access to the vascular compartment. The three diarrheal strains, 11168, 79-193 and 81-176, were substantially more susceptible to human serum, with greater than 1 to 7 \log_{10} increased killing compared to the two systemic strains 84-19 and 84-25 ($p < 0.02$) (Fig. 6A). Since removal of sialic acid residues from the distal LOS in *C. jejuni* strains 81-176 and MSC57360 increased its susceptibility to the bactericidal activities in normal human serum (NHS),^{8,9} we asked whether the distal LOS may play a similar role in systemic *C. jejuni* strains. However, there were no significant differences between wild-type 84-19 and 84-25 and their respective isogenic *waaF* mutants, lacking the outer-core of LOS (Fig. 6B). Next, to assess the role of capsule in mediating the relative serum-resistance of the systemic strains, we examined survival of the wild-type and capsule mutants of the systemic strains in the presence of NHS (Fig. 6B). Compared to the wild-type strains, the capsule mutants of 84-19 and 84-25 showed 4–5 \log_{10} increased serum-dependent killing. Complementation of the 84-25 mutant with *kpsMT* in trans in *hipO* restored the serum-resistant phenotype of this strain. As a control, insertion of a *cat* cassette into *hipO* showed no loss of serum resistance compared with wild-type. In summary, these results show that while capsule contributed substantially to serum resistance of the systemic strains, LOS chain length did not.

Discussion

In this work, we sought to expand the understanding of the host-microbe interactions that affect the pathogenesis of *C. jejuni*, an important but under-studied human pathogen. We focused on the interplay between components of the host's innate immune system and *C. jejuni*'s oligosaccharide surface structures, using a systemic (84-25) isolate. We hypothesized that this virulent strain might possess unique surface characteristics that assisted its apparent ability to evade not only mucosal/luminal defenses

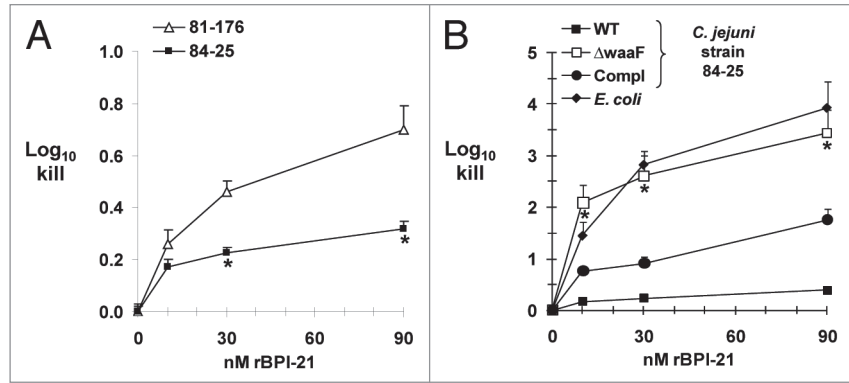


Figure 5. Killing of *C. jejuni* strains by the recombinant N-terminal fragment of human Bactericidal/Permeability-Increasing Protein (rBPI-21). (A) The *C. jejuni* systemic strain 84-25 or the diarrheal strain 81-176 were incubated with increasing doses of rBPI-21 or buffer as indicated. * $p < 0.0001$ vs. the diarrheal strain 81-176. (B) The wild-type strain of *C. jejuni* 84-25 (WT), the LOS mutant (*waaF*), the complemented strain (*waaF/comp*) or the control strain *E. coli* K1/r were incubated with rBPI-21 as in (A). All data are the mean + SD. 3–8 experiments performed in duplicate to quadruplicate. * $p < 0.005$ for *C. jejuni waaF* vs. WT or complement.

(i.e., CAPs) but also systemic defenses such as complement and the neutrophil-derived antimicrobial protein BPI. With regard to LOS, in silico analysis (Fig. 2) suggested that strain 84-25 likely expresses an LOS inner-core structure similar to the diarrheal strains we tested. Based on this preliminary evidence, truncations of the LOS outer-core might therefore provide insights into the basis for altered susceptibility to innate defenses.

To address these questions, we constructed isogenic capsule and LOS mutants and their respective complements (Figs. 1 and 3). While complementation of *C. jejuni* has been challenging since many strains are refractory to the introduction of exogenous genes probably due to restriction barriers,⁴⁹⁻⁵¹ we have devised a chromosomal approach that overcomes limitations associated with shuttle vectors or previously published methods^{8,11,28-30} through novel use of the *hipO* locus. Compared to using one of the ribosomal RNA loci (*rpsL*) for complementation,²⁸ the single-copy *hipO* locus allows precise identification of the chromosomal location of the exogenous gene cassette. In addition, the *hipO* locus is not as prone to recombination as are the multiple *rpsL* loci.²⁸ Finally, the ubiquity of the *hipO* locus²⁷ provides a complementation site for nearly all *C. jejuni* strains compared to loci found only in some strains such as the arylsulfatase locus.²⁹

Introduction of the *kpsMT* genes into the non-essential *hipO* locus on the *C. jejuni* chromosome yielded partial restoration of capsule and full-length LOS expression. Such partial complementation may have occurred if regulation of *waaF* and *kpsMT* in the *hipO* locus was not identical to that in their native loci, perhaps leading to diminished transcription. Nonetheless, the degree of functional complementation that we could achieve facilitates manipulation of this important microorganism that has low genetic tractability in general.

To assess the contribution of capsule and full-length LOS to resistance to innate immune defenses, we measured the bacterial killing of wild-type, mutant and complemented strains of the systemic strain 84-25 after exposure to cationic antimicrobial

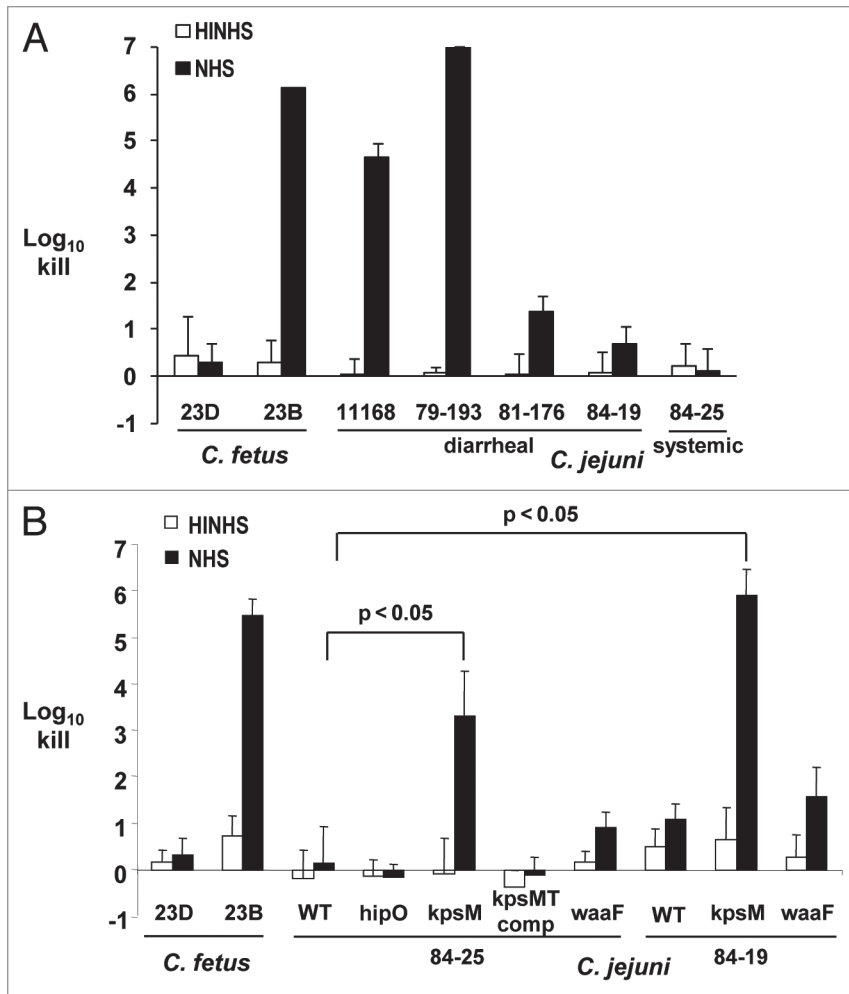


Figure 6. Complement-dependent killing of *Campylobacter* strains by normal human serum. The indicated *Campylobacter* strains were incubated with 10% serum (NHS, normal human serum; black bars) or serum previously heated to inactivate complement (HINHs, heat-inactivated NHS; white bars). *C. fetus* strains 23B and 23D are the serum-sensitive and -resistant controls, respectively. (A) Susceptibility of *C. jejuni* diarrheal (11168, 79-193 and 81-176) and systemic strains (84-25 and 84-19) to 10% NHS versus control (HINHs). (B) Susceptibility of *kpsM* and *waaF* mutants in *C. jejuni* systemic strain backgrounds (84-25 and 84-19). Bars represent the mean + SD for at least three replicate experiments. Brackets indicate statistically significant differences in survival between wild-type and *kpsM* mutants ($p < 0.05$) using the Student's two-tailed t-test.

peptides and a protein, rBPI-21, examples of innate immune defenses that *C. jejuni* would encounter during host infection. As an initial approach to testing *C. jejuni* sensitivity to CAPs, we measured its sensitivity to polymyxin B, a clinically useful antibiotic that also is a cationic, lipid A-interactive peptide (Table 3). Because polymyxin B shares these attributes with endogenous CAPs, it may be used as an inexpensive and easily obtained surrogate marker for CAP sensitivity. The four- to six-fold increased polymyxin B susceptibility of two, LOS-truncated systemic strains (84-25 and 84-19) relative to the isogenic wild-type strains (Table 3) supports the hypothesis that the distal LOS limited access to lipid A, the target of polymyxin B bioactivity.⁵² The equal susceptibility of the wild-type strain and its acapsular

mutant suggests that the sugars present in the capsule do not provide similar protection from polymyxin B.

Extending this observation to naturally-occurring CAPs produced by Paneth cells of the intestinal epithelium, we found that both the human α -defensin and its murine homolog Crp4 (Fig. 4A) produced substantially greater killing of the LOS truncated mutant compared to the wild-type strain. In these experiments, the $\sim 1 \log_{10}$ excess killing of the LOS mutant in the presence of buffer alone likely reflects the more fragile nature of this strain which became manifest over the extended time course (120 min) of these experiments. Nonetheless, the approximately ten-fold increased killing of the LOS mutant by human α -defensin-5 was significantly greater ($p < 0.05$) compared to buffer alone (Fig. 4A). Overall, our data showing that the LOS truncated mutant had increased susceptibility to killing by α -defensins (Fig. 4A) compared to the wild-type strain supports the hypothesis that sugars present in the distal LOS confer a level of protection against mucosal innate immune defenses.

C. jejuni strains that withstand these defenses have the opportunity to gain access to the vascular space and cause systemic infection.¹² Among the multiple innate immune effectors present in blood, neutrophils and complement are key cellular and soluble components, since when they are present in insufficient levels or function aberrantly, therefore serious clinical sequelae.^{23,53,54} After exposure to a mixture of neutrophil granule-derived peptides and proteins (hNGP), the enhanced killing of the LOS-truncated mutant compared to the wild-type strain indicates that full-length LOS is necessary for protection against hNGP (Fig. 4B). This result was particularly striking given the low concentration of hNGP extract used. Since neutrophils are the major source of the cathelicidin family of CAPs,⁴¹ we also measured bacterial survival after exposure to LL-37 (Fig. 4C). The greater than 1,000-fold difference in killing between the LOS-truncated mutant and both the wild-type and complemented strains suggests that the distal LOS sugars are an impediment to the binding of this CAP to lipid A, thereby providing protection against CAP-dependent killing. The similar pattern of results obtained with the chicken cathelicidin homolog fowlicidin-1 (Fig. 4D) indicates that *C. jejuni* uses the same strategy to evade poultry innate immune defenses. Although our results with human and chicken cathelicidin were similar, further study of poultry innate immunity may reveal differences that underlie why *C. jejuni* is a commensal in poultry and a pathogen in humans.

In addition to being the major source of the host's cathelicidins, neutrophils are also the primary source of BPI, which is lethal to most strains of Gram-negative bacteria via its high affinity for the lipid A portion of LPS.^{45,55} BPI accumulates to levels as high as 1 µg/ml in sites of inflammation and infection.⁵⁶⁻⁵⁸ Rough (i.e., short-chain) LPS chemotypes are relatively more sensitive to BPI-mediated killing than are bacteria expressing smooth or long-chain LPS, although the smaller, N-terminal recombinant fragment represented by rBPI-21 seems less subject to this apparent steric hindrance.⁴⁷ rBPI-21 dose-dependent increased killing of the diarrheal strain versus the systemic strain 84-25 (Fig. 5A) suggests that differences in the surface structures of these two strains contribute to their disparate susceptibility to innate defenses. Our finding that BPI mediated increased, potent and dose-dependent killing of the LOS truncated mutant versus the wild-type strain is consistent with the results obtained with hNGP and CAPs (Fig. 5B) indicating that *C. jejuni* 84-25 requires expression of distal LOS residues to evade the antibacterial effect of CAPs and BPI. Whether or not this effect is due to steric hindrance imparted by the distal LOS, involves binding of CAPs/BPI to sialic acid residues in the distal LOS region and away from their targets on lipid A, differences in other surface structures including capsular composition, or a combination of these factors, is unknown. Our *in silico* analyses of the LOS biosynthetic loci suggest that the inner-core LOS of the diarrheal strains 11168 and 81-176 are identical, and that the outer-cores are similar. Since *waaF* mutation would lead to loss of the outer-core sugar residues including negatively-charged sialic acid, it is intriguing to speculate that sialic acid may act as a charge decoy leading to binding of CAPs and BPI at sites distant from lipid A on LOS. However, the mechanism of relative BPI-resistance afforded by distal LOS sugar residues in *C. jejuni* requires further study.

Complement-mediated bacterial killing is a key component of the humoral arm of innate immunity necessary to control systemic infection from mucosal sources. Encapsulated bacteria thwart the antibacterial effect of complement by preventing deposition of the membrane attack complex on bacterial membranes.⁵⁹ That two systemic *C. jejuni* strains (84-25 and 84-19) were more resistant to complement-mediated killing compared to diarrheal strains (Fig. 6A) suggests that the capsules of these strains may have contributed to both their survival in the vascular compartment and ability to cause systemic disease. Although strains 11168 and 84-25 are both of the HS2 Penner serotype, their markedly different serum sensitivities may suggest that substituent modifications,^{60,61} which also may confer resistance to antimicrobial peptides, may not be sufficiently antigenic to be distinguished by Penner anti-sera.

Next, capsule mutants of both systemic strains were >1,000-fold more sensitive to complement-mediated killing compared to their respective wild-type strains (Fig. 6B). Interestingly, the LOS truncated mutants of *C. jejuni* 84-25 and 84-19 did not show increased susceptibility to complement-mediated killing (Fig. 6B), whereas an LOS mutant of another *C. jejuni* strain (MSC57360) lacking distal sialic acid residues became serum-sensitive.⁸ We speculate that differences in the chemical

composition and physical presentation of the sugar residues in LOS MSC57360 versus 84-25 and 84-19 may afford differing resistance to complement-mediated killing; this unanswered question requires further study. As speculated with regard to BPI susceptibility, the presence of sialic acid alone may not be as important as the spatial configuration of those residues in the context of capsule versus LOS. Finally, it is important to note that bacterial virulence factors other than capsule or full-length LOS (such as secretion systems/effector, outer membrane proteins, etc.) also likely contribute to the phenotypic differences between systemic and diarrheal *C. jejuni* strains.

In summary, we report the construction of capsule and LOS mutant strains of two systemic *C. jejuni* strains, and the development of a chromosomal-based complementation strategy utilizing the non-essential *hipO* locus. Our results with these mutants show that the distal LOS of these systemic *C. jejuni* strains contribute to resistance to CAPs and BPI, whereas capsule contributes to complement resistance. The combination of the specific distal LOS and capsule composition of these systemic strains contribute to the phenotype of greater resistance to innate defenses versus diarrheal isolates, allowing their evasion of the usually effective luminal, mucosal and systemic innate immune mechanisms.

Materials and Methods

Strains and growth conditions. The bacterial strains used in this study (Table 1) were obtained from the NYU Campylobacter/Helicobacter strain collection in our laboratory. *E. coli* XL-1blue was used for construction and cloning of plasmids and was grown in Luria-Bertani media at 37°C. *C. jejuni* cells were routinely cultured on Trypticase soy agar (TSA) plates with 5% sheep blood for 2–3 d at 37°C in a 5% CO₂ atmosphere. Campylobacter strains were stored at -80°C in Brucella broth supplemented with 15% glycerol.

Construction of *kpsM* mutants. To construct the *kpsM* mutant, amplification of *kpsM* was performed with oligonucleotides cj1449F23 and kpsTR640 (Table 2) according to a standard protocol with designated primers, as described in reference 62. The 3 kb PCR product was gel-purified and ligated to pGEM-T-Easy, producing pTK800. Unique *Bam*HI sites were introduced into *kpsM* contained within pTK800 through inverse PCR with primers kpsMF481bam and kpsMR320bam, and a kanamycin resistance (*aphA*) cassette flanked by *Bam*HI recognition sites was ligated to generate pTK802. This suicide vector (pTK802) was used to transform cells of *C. jejuni* strains 84-19 and 84-25 by electroporation, as described in reference 50. Transformants were confirmed by PCR to have insertion of the *aphA* cassette in the *kpsM* locus.

Construction of *waaF* mutants. A *C. jejuni* mutant expressing a truncated LOS, *waaF*, encoding a heptosyltransferase necessary for the addition of the second heptose beyond KDO,³⁵ was constructed using insertional mutagenesis. First, amplification of *waaF* was performed with oligonucleotides gmhaF3b and wlasaR3 (Table 2), as described in reference 35. PCRs were performed according to a standard protocol with designated primers

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or ref.
Strains		
<i>Campylobacter jejuni</i>		
11168	Wild-type strain, diarrheal isolate	68
81-176	Wild-type strain, diarrheal isolate	69
79-193	Wild-type strain, diarrheal isolate	12
84-25	Wild-type strain, HS:2, meningitis isolate	12
84-25 <i>kpsM</i>	<i>kpsM</i> with <i>aphA</i> insertion	This work
84-25 <i>kpsM</i> /comp	<i>kpsM::aphA</i> complemented with <i>kpsMT</i> in <i>hipO</i> locus	This work
84-25 <i>waaF</i>	<i>waaF</i> with <i>cat</i> insertion	This work
84-25 <i>waaF</i> /comp	<i>waaF::cat</i> complemented with <i>waaF</i> in <i>hipO</i> locus	This work
84-19	Wild-type strain, meningitis isolate	12
84-19 <i>kpsM</i>	<i>kpsM</i> with <i>aphA</i> insertion	This work
84-19 <i>waaF</i>	<i>waaF</i> with <i>cat</i> insertion	This work
<i>Campylobacter fetus</i>		
23D	Wild-type strain, serum-resistant	70
23B	23D, with <i>sapA</i> promoter deletion	70
Plasmids		
pTK802	<i>kpsM::aphA</i> in pGEMT-Easy	This work
pTK1106	<i>waaF::cat</i> in pGEMT-Easy	This work
pTK1311D	<i>hipO::waaF-aphA</i> in pGEMT-Easy	This work
pTK1330	<i>hipO::kpsMT-cat</i> in pGEMT-Easy	This work
pTK1301B	<i>hipO::cat</i> in pGEMT-Easy	This work
pTK1301D	<i>hipO::aphA</i> in pGEMT-Easy	This work
pTK1302B	<i>hipO::kpsM-cat</i> in pGEMT-Easy	This work

(Table 2), as described in reference 35. The 3 kb *waaF* PCR product was gel-purified and ligated to pGEM-T-Easy, producing pTK1100. Unique *Bam*HI sites were introduced into *waaF* contained within pTK1100 through inverse PCR with primers *waaFR490bam2* and *waaFF550bam2*, and a chloramphenicol acetyltransferase (*cat*) cassette flanked by *Bam*HI recognition sites was ligated to generate pTK1106. This suicide vector (pTK1106) was used to transform cells of *C. jejuni* strains 84-19 and 84-25 by electroporation, as described in reference 50. Transformants were examined by PCR using primers *gmhaF3b* and *pBSC103Fbam* to confirm insertion of *cat* into *waaF*. Retention of capsule expression in candidate LOS mutants was confirmed by Alcian Blue staining of Tricine-SDS PAGE bacterial lysates.

Complementation of *kpsM* and *waaF* mutants. Chromosomal DNA from *C. jejuni* strain 84-25 was extracted with the Wizard Genomic DNA Purification kit (Promega, Madison, WI). PCR amplification of a 3 kb fragment containing *hipO* and 1 kb of flanking sequence was performed with oligonucleotides designated Cj0984F646 and Cj0987F913 (Table 2). The amplified product was cloned into pGEM-T Easy (Promega), yielding pTK1300. Unique *Bam*HI and *Mfe*I restriction sites were introduced into *hipO* by inverse PCR, using oligonucleotides

hipOR565bam and *hipOF574mfeB*. The inverse PCR product was digested with *Bam*HI and *Mfe*I, and then *aphA* was inserted into *hipO* to create pTK1301-D. PCR primers *waaFpr1bamF* and *waaFpr1bamR* were used to amplify *waaF* with its 73 bp upstream (intergenic) sequence that includes its likely promoter. The PCR-amplified product was purified using a Qiagen PCR purification kit, then digested with *Bam*HI, and the product cloned into pTK1301-D and screened by PCR with primers Cj0984F681 and Cj0987F913; the plasmid containing *waaF* was designated pTK1311-D. Cells of *C. jejuni* mutant strain 84-25 *waaF* were electroporated with pTK1311-D and transformants selected for growth on Brucella agar containing kanamycin and chloramphenicol. Hippuricase-negative transformants were confirmed by PCR using primers Cj0984F339 and *waaFF1*, to show introduction of *waaF* into the *hipO* locus by allelic exchange.

PCR primers *kpsMpr1bamR* and *kpsER120bam* were used to amplify the *kpsMT* genes; the resulting PCR product, which includes 126 bp of flanking sequence upstream of *kpsM*, was cloned into the *Bam*HI site of pTK1301-B to produce pTK1330. The *C. jejuni* *kpsM* mutant was electroporated with the appropriate complementation plasmids, pTK1302B or pTK1330, and transformants were selected for growth on Brucella agar containing kanamycin and chloramphenicol. Hippuricase-negative transformants were confirmed by PCR for allelic exchange in the *hipO* locus with oligonucleotides Cj0984F339 and *kpsMF1*.

Detection of capsule and LOS expression. Wild-type and mutant *C. jejuni* cells were harvested following 48 h growth as previously described, washed in PBS, standardized to an optical density at 600 nm (OD₆₀₀) of 2.0, then solubilized in 200 µl of sample buffer (2% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris-Cl, pH 6.8 and 10 mg bromophenol blue), at 100°C for 10 min, and then incubated with 60 µg proteinase K for 1 h at 60°C. The whole cell lysates were fractionated on 12% SDS-PAGE or 15% Tricine sodium-dodecyl sulfate polyacrylamide gels at 35 mA for 10 h. Prestained protein markers were used (Biorad, Hercules, CA), and bands resolved with dual silver and Alcian Blue stains, as described in reference 63.

Determination of growth inhibition by antibiotics. To determine susceptibilities of *C. jejuni* to antimicrobial agents, the Steer's replicator agar dilution procedure was repeated three or more times as described in reference 64. Briefly, bacteria were suspended in PBS and distributed in 150 µl aliquots in microtiter plate wells. A Steer's replicator was used to inoculate bacteria in quadruplicate onto Brucella agar supplemented with indicated amounts of antibiotics. The antimicrobial concentrations (µg/ml) were as follows: polymyxin B (0.5–50), streptomycin (0.5–10), nalidixic acid (1–20). Plates were incubated at 37°C for 3 d, and examined for growth. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic that resulted in complete inhibition of growth. All determinations were done at least in duplicate.

Susceptibility of *C. jejuni* strains to CAPs and BPI. Human neutrophil granule protein acid extract (hNGP) was obtained from Yvette Z. Weinrauch (NYU School of Medicine, New York, NY),²¹ LL-37 was obtained from Panatecs (Tubingen, Germany),

Table 2. Oligonucleotide primers used in this study

Primer designation	Genomic location ^a	Primer Sequence (5'->3') ^b	ORF	Orientation
gmhaF3b	1082341–1082367	TAC CCA AAT CGC TAA AGT AGG TGA GC	gmha	R
wlasaR3	1080361–1080392	AAA CTG CAG CAC TTA GCC CAA ACC GAC CAG C	waaV	F
waaFR490bam2	1081397–1081317	CGC <u>GGA TCC</u> GCG TTC CAA AGC TTG CAC CAG GGT TGA G	waaF	R
waaFF550bam2	1081458–1081482	CGC <u>GGA TCC</u> GCG ATG ATA TTT TAA TCT TTG GTG CAG G	waaF	F
cj0984F339	918174–918196	TAA TCT TTT TGG CAT TGT AAG G	Cj0984	F
cj0987F913	920041–920019	TTA GTA TTG ATA CAG ATT TTT GC	Cj0987	R
hipOR565bam	919168–919190	CGC <u>GGA TCC</u> GCG TTC AAT ACT ATA ACT ATC CGA AG	hipO	R
hipOF574mfeb	919131–919110	AAC <u>AAT TGT</u> TAA GGC AAA AGA TCC TAT TTA TG	hipO	F
waaFpr1bamF	1081867–1081844	CGC <u>GGA TCC</u> GCG TCA TAG ATG AGA GTT TTT AAG TAA	waaV	F
waaFpr1bamR	1080854–1080882	CGC <u>GGA TCC</u> GCG TTC CTA AAT TTT GTT AAA ATA ATA AAA AC	gmhA	R
cj0984F681	918516–918534	ACT CAA AGA AAT TCA AAT C	Cj0984	F
pBSC103Fbam	NA ^c	CGC <u>GGA TCC</u> GCG ATC GTA TGG AGC GGA CAA CG	cat	F
waaFF1	1080908–1080930	ATG AAA ATT TTT ATA CAT CTT CC	waaF	F
cj1449F23	1388207–1388232	TTA GAA TTT ATA AAA AAT GAG CAG C	Cj1449	F
kpsTR640	1385624–1385652	TAT TCC TTC ATC TAC ATC ATC ATA AAC C	kpsT	R
kpsMF481bam	1387224–1387201	CGG <u>GGA TCC</u> CGA TTA TTT GGC ATT TTG TGG AAC C	kpsM	F
kpsMR320bam	1387385–1387410	CGG <u>GAT CCC</u> GTT CTA GCA ATA AAT ACA TGT ATA GG	kpsM	R
Cj0984F646	918842–918501	AAA GAG CTT TTA GCA AAC C	Cj0984	F
kpsMpr1bamR	1387775–1387825	CGG <u>GAT CCC</u> GAA CAA TGC TTT AGG ACT TAG	Cj1449	R
kpsER120bam	1386144–1386157	CGG <u>GAT CCC</u> GTG GTG CTG CAA TC	kpsE	F
kpsMF1	1387679–1387699	ATG TTA AAT GTA ATT TAT GC	kpsM	F

^aLocation based on the sequence of *C. jejuni* strain 11168. ^bRestriction sites underlined; *Bam*HI (GGATCC) and *Mfe*I (CAATTG). ^cNot applicable.

and fowlicidin-1 was a kind gift from Guolong Zhang (Oklahoma State University, Stillwater, OK). The α -defensin murine cryptidin-4 (Crp4) and human β -defensin-5 were kindly provided by Andre J. Ouellette (University of California, Irvine, CA). A 21 kDa recombinant N-terminal fragment of the Bactericidal/Permeability-Increasing Protein (rBPI-21), which retains all the bactericidal and LPS-neutralizing bioactivities of the holoprotein,⁴⁵ was kindly provided by Dr. Jerrold Weiss (University of Iowa School of Medicine, Iowa City, IA).

In assays testing CAPs and hNGP, *C. jejuni* strains were harvested after 48 h of growth on TSA plates at 37°C under micro-aerophilic conditions and resuspended in Hank's balanced salt solution (without calcium or magnesium, supplemented with 0.3% casamino acid and 10 mM HEPES pH 7.4) to a final concentration of 10⁸/ml. Bacteria were incubated with hNGP (5% vol/vol), fowlicidin-1 (10 μ g/ml), LL-37 (10 μ g/ml) or diluent (20 mM sodium acetate, pH 4) in a total volume of 100 μ l at 37°C. Fifty μ l aliquots were serially diluted in phosphate buffered saline and inoculated on TSA plates. For the rBPI-21 assay, *C. jejuni* strains were harvested as described above and resuspended in Hank's balanced salt solution (without calcium or magnesium, supplemented with 0.5% pyrogen-free human serum albumin and 10 mM HEPES pH 7.4) to a concentration of 1.1 x 10⁶/ml in a volume of 90 μ l. Bacteria were incubated with 10 μ l of rBPI-21 or diluent (10 mM sodium acetate, pH 4.0) in a final volume of 100 μ l at 37°C x 1 h. For all assays, at the stated intervals, aliquots were serially diluted in PBS, inoculated on TSA plates, incubated at 37°C in 5% CO₂ for three days, and

Table 3. Susceptibility of *C. jejuni* strains to antimicrobial agents

Strain	MIC (μ g/ml) ^a		
	Polymyxin B	Streptomycin	Nalidixic acid
79-193	10	ND ^b	ND
81-176	3	ND	ND
84-25	30	5	2
84-25 kpsM	30	5	2
84-25 kpsM/comp	30	ND	ND
84-25 waaF	5 ^c	5	2
84-25 waaF/comp	20 ^c	5	2
84-19	20 ^d	5	2
84-19 waaF	5 ^d	5	2

^aGrowth is shown as measured in agar dilution MIC assays, using an inoculum of 10⁷ *C. jejuni* cells/ml. Results are the mean of two or more determinations of four replicates each. ^bND not determined. ^{c,d}Statistically significant differences (p < 0.005) using the Student's two-tailed t-test.

the number of colony forming units (cfu) counted. Log₁₀ kill was determined by subtracting the difference between the number of cfu before and after exposure to CAPs, hNGP, rBPI-21 and their respective diluents.⁶⁵

Serum bactericidal assays. Normal human serum (NHS) was obtained from donors who had tested negative for anti-*C. jejuni* antibodies by ELISA, as described in reference 66. Fresh serum from three donors was pooled, sterilized by passage through a 0.22 μ m filter, and stored at -70°C.

C. jejuni strains were harvested after 48 h growth on TSA as previously described, washed in sterile saline and resuspended from 10¹/ml to 10⁶/ml in Medium 199 with Hank's balanced salt solution (with 0.01% glutamine). From each dilution, 150 µl aliquots of bacteria were transferred in triplicate to microtiter wells. To each well, 50 µl was added of either 40% NHS in Medium 199, 40% heat-inactivated (56°C for 30 min) NHS (HINHS) or Medium 199 (buffer alone). When present, the final serum concentration was 10%. After 60 min of incubation, 50 µl aliquots from the wells were inoculated onto TSA plates. The plates were incubated at 37°C in 5% CO₂ for four days, and colony forming units counted. Log₁₀ kill was determined by subtracting the difference between the number of cells before and after exposure to NHS or HINHS.⁶⁵ Capsule and LOS expression in strain 81-176 was confirmed by Alcian Blue and silver staining (Sup. Fig. 1).

In silico analyses of *C. jejuni* LOS loci. Nucleotide sequences of *C. jejuni* strains 81-176 (NC_008787.1), 84-25 (NZ_AANT00000000.2) and 11168 (NC_002163.1) were obtained from NCBI Entrez genome (www.ncbi.nlm.nih.gov/projects/genome/?db=genome). Identification of orthologs

was performed with the Basic Local Alignment Search Tool (BLAST) proGram for microbial genomes (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) and confirmed with ClustalX 2.0.12 analysis.⁶⁷

Statistical analyses. The Student's t-test was used in comparisons of strain characteristics, with p value < 0.05 considered significant.

Acknowledgements

We are grateful to Yvette Z. Weinrauch and Andre J. Ouellette (University of California, Irvine) for advice and reagents. This work was supported in part by grant R01 AI24145 from the National Institutes of Health, by the Medical Research Service of the Department of Veterans Affairs, by the Diane Belfer Program in Microbial Ecology and by the Saperstein Medical Scholars Program.

Note

Supplemental materials can be found at: www.landesbioscience.com/journals/virulence/article/14752

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