Characteristics of an avirulent *Campylobacter jejuni* strain and its virulence-enhanced variants

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Summary. The virulence of *Campylobacter jejuni* for 11-day-old chick embryos is associated with the ability to invade the chorio-allantoic membrane, to resist phagocytosis and to survive and proliferate *in vivo*. The pathogenicity of a well characterised avirulent *C. jejuni* strain was enhanced by passaging it intravenously and chorio-allantoically through chick embryos. The resulting isogenic variants had greatly increased ability to survive *in vivo*. In this study, the morphological and cell-surface characteristics of the avirulent parental strain were compared with those of the more virulent variants to determine whether pathogenicity was associated with one or more cell-surface constituents. Changes associated with the increased virulence of the two variants included alterations in cultural and cellular morphology, loss of flagella, expression of a new outer-membrane protein, alterations in cell-surface carbohydrates and decreases in cell-surface hydrophobicity.

Introduction

Campylobacter jejuni is an important enteric pathogen in man. Its pathogenicity is incompletely understood, but tissue invasion and toxin production probably play a role in disease.^{1,2} As with other pathogenic micro-organisms, the cell surface of *C. jejuni* interacts directly with the human host, leading to infection and the overwhelming of host defence mechanisms. Structures on the cell surface of *C. jejuni* of potential pathogenic significance include flagella, lipopolysaccharide (LPS) and outer-membrane proteins (OMPs).

C. jejuni is motile by means of a single polar flagellum, and motility facilitates colonisation of the mucous lining of the gastrointestinal tract.^{3,4,5} There is some evidence that flagella also function as adhesins, enabling campylobacters to attach to the epithelial cell surface.^{6,7} Recently, both phase⁸ and antigenic⁹ variation of campylobacter flagella have been reported. Either type of variation may enable the bacterium to avoid the immune response of the host.

In many bacteria, LPS and OMPs play important roles in attachment, invasion, serum resistance, resistance to phagocytosis and the acquisition of iron.¹⁰ The composition of the outer membrane of *C. jejuni* has been the subject of several studies.^{11–23} Little is known about its potential importance in pathogenesis, but LPS⁶ and several OMPs^{24,25} have been implicated as adhesins.

The 11-day-old chick embryo is a useful *in-vivo*

model for the analysis of certain virulence factors in *Campylobacter* strains.^{26,27} Virulence in this system is associated with the ability to invade the chorio-allantoic membrane (CAM), to resist ingestion by phagocytic cells, and to survive and multiply *in vivo*.^{26,27} In this study, the morphological and cell-surface characteristics of a non-invasive avirulent *C. jejuni* strain and its two, *in-vivo* passaged, more virulent variants were analysed to determine any possible relationship to pathogenicity.

Materials and methods

Bacterial strains and cultural conditions

The isolation and characterisation of *C. jejuni* LOPEZ, a human faecal isolate that is avirulent for 11-day-old chick embryos, and the derivation of the more virulent, isogenic variants, LOPEZ-3 and LOPEZ-8, by passage through such embryos, have been described previously.^{26,27} Strains were grown on brucella agar with defibrinated sheep blood 5% at 37°C.²⁷ After incubation for 24 h, the growth was harvested and used to inoculate brucella broth-brucella agar biphasic culture medium contained in Erlenmeyer flasks.²⁷ The flasks were incubated micro-aerobically without shaking at 37°C.²⁷

Salmonella typhimurium SL428 (semi-rough; SR) and *S. typhimurium* SL3749 (chemotype Ra) were kindly provided by Dr K. E. Sanderson, Salmonella Genetic Stock Centre, University of Calgary, Calgary, Alberta, Canada. *Salmonella* strains were grown aerobically in brucella broth at 37°C for 24 h.

Electronmicroscopy

Middle to late log-phase cells grown in biphasic medium were fixed with glutaraldehyde 1.5%, stained with uranyl acetate 1.5%, and examined with a JEOL 100 CX electronmicroscope.

Hydrophobic interaction chromatography

The method of Smyth *et al.*²⁸ was used to compare the surface hydrophobicity of *C. jejuni* strains. Briefly, sterile pasteur pipettes were packed by gravity feed to a height of 30 mm with Octyl Sepharose C1-4B (Pharmacia). Gel beds were washed with 10 ml of 1 M ammonium sulphate buffered with 10 mM sodium phosphate, pH 6.8, (buffer A). The entire chromatography procedure was performed at room temperature.

Middle to late log-phase cells grown in biphasic medium were harvested by centrifugation and resuspended in buffer A to a turbidity of 500 Klett units (green filter). Portions (100 µl) of the bacterial suspension were placed on the gel beds and in a control tube containing 5 ml of buffer A. Gel beds were washed with 5 ml of buffer A and the eluates were collected in sterile tubes. The number of cfu present in each eluate and in the control tubes was determined by plating serial 10-fold dilutions. The percentage (mean value of three samples) of adsorbed bacteria was calculated by the following formula: (cfu in the control – cfu in the eluate)/(cfu in the control) × 100. Each strain was tested in triplicate, and experiments were repeated once.

Preparation of outer membranes

C. jejuni cells were fractionated to prepare outer membranes by a procedure previously shown to yield outer membranes uncontaminated by other cell constituents.²⁹ Briefly, cultures of middle to late log-phase cells grown in biphasic medium were harvested by centrifugation, and the cell pellets were frozen at –20°C for at least 24 h. The procedure of Inouye and Guthrie³⁰ was used to prepare total cell envelopes, as follows. The cells (1 g, wet weight) were resuspended in 10 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 5 mM MgSO₄. DNAase I (Sigma) was added to a concentration of 10 µg/ml, and cells were disrupted ultrasonically. The preparation was centrifuged at low speed to remove unlysed cells. The total membrane fraction was collected by centrifuging the supernate at 100000 *g* at 20°C for 45 min (L8-M ultracentrifuge; Beckman Instruments Inc., Fullerton, CA, USA). Outer membranes were prepared by the method of Filip *et al.*,³¹ in which treatment with Sarkosyl (Ciba-Geigy Corp., Summit, NJ, USA) 1% for 30 min was used to solubilise the inner membrane.

SDS-PAGE of outer membranes

Outer membranes were solubilised in Laemmli solubilisation buffer,³² heated at 100°C for 5 min, and

the polypeptides were separated by the SDS-PAGE system of Ames³³ as described previously.²⁹ Stacking and separating gels consisted of acrylamide 5% w/v and 10% w/v, respectively. Gels were stained with Coomassie Brilliant Blue R (Sigma), destained and dried. Protein standards were: phosphorylase b (94 kDa), albumin (67 kDa), catalase (60 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) (Pharmacia Fine Chemicals, Piscataway, NJ, USA).

LPS analysis

Proteinase K-digested whole cell lysates were used as the source of LPS for analysis by SDS-PAGE. Lysates were prepared by a modification of the procedure described by Kimura and Hansen.³⁴ Briefly, middle to late log-phase cells grown in biphasic medium were harvested by centrifugation and the pelleted cells were resuspended in phosphate-buffered saline (PBS). This suspension was centrifuged at 7000 *g* for 10 min and the cell pellet was resuspended in PBS. The bacteria were solubilised by adding 0.5 volume of 3 × concentrated digestion buffer (SDS 6% w/v, glycerol 30% v/v, 0.1875 M Tris-HCl, pH 6.8, and Pyronin Y tracking dye (0.015% w/v)). Lysates were heated at 100°C for 10 min, divided into multiple small volumes and stored at –20°C for a maximum of 2 weeks.

For LPS analysis, a 20-µl portion of each lysate was diluted with 20 µl of digestion buffer consisting of SDS 0.1% w/v, glycerol 10% v/v, 0.0625 M Tris-HCl, pH 6.8, and Pyronin Y 0.005% w/v. Proteins were digested by adding 10 µl of a digestion buffer containing proteinase K (Sigma) 2 mg/ml and incubating for 1 h at 60°C. All samples were heated at 100°C for 5 min immediately before being loaded into the wells for electrophoresis. Purified LPS from the Ra and Rb chemotypes of *S. minnesota* was purchased from List Biological Laboratories Inc., Campbell, CA, USA.

LPS preparations were analysed by discontinuous SDS-PAGE³³ with the Laemmli buffer system³² as described by Kimura and Hansen.³⁴ The separating gel consisted of a linear polyacrylamide gradient 10–15% w/v containing a linear glycerol gradient 0–37.5% v/v. The stacking gel contained polyacrylamide 4% w/v. Electrophoresis was performed at 4°C with a 20 mA constant current. After electrophoresis, the gels were fixed, and the LPS was visualised by the silver stain technique of Tsai.³⁵

Lectin agglutination

The sources of 14 lectins contained in panels (E.Y. Laboratories, San Mateo, CA, USA) were: *Canavalia ensiformis* (concanavalin A), *Lens culinaris* (lentil lectin), *Solanum tuberosum* (potato starch agglutinin), *Triticum vulgare* (wheat germ agglutinin), *Griffonia simplicifolia* II, *Dolichos bifloris*, *Helix pomatia*, *Glycine max* (soybean agglutinin), *Arachis hypogaeae* (pea-

nut agglutinin), *Griffonia simplicifolia* I, *Trichosanthes kinlowii*, *Ulex europaeus* I, *Limax flavus* and *Phaseolus vulgaris*. The specificities of these lectins have been described previously.^{36,37} Bacterial interaction with the lectins was tested as follows: *C. jejuni* strains were grown in biphasic medium and harvested by centrifugation. Cell pellets were resuspended in 10 ml of Tris-buffered saline (10 mM Tris, 0.13 M NaCl, pH 7.4; TBS). The cultures were heated at 100°C for 1 h to enhance their reactivity with lectins and to eliminate non-specific agglutination reactions.³⁸ The heated cells were washed once with TBS and then resuspended in TBS containing DNAase I (Sigma) 10 µg/ml to a turbidity of 180 units (10^9 – 3×10^9 organisms/ml) as measured in a Klett-Summerson colorimeter (Klett Manufacturing Co., NY, USA) fitted with a green filter. Cell suspensions were then checked for auto-agglutination under a dissecting microscope. Bacterial suspension (100 µl) was added to each lectin well, and the lectin panels were rotated (80 rpm) on a shaker. Cell agglutination was examined under a dissecting microscope after 5, 15 and 30 min.

Statistical analysis

Statistical significance was determined by the Student's *t* test.

Results

Virulence of *C. jejuni* strains and passaged variants for chick embryos

Previous reports described the virulence of human isolates for 11-day-old chick embryos inoculated *via* the chorio-allantoic and intravenous routes.^{26,27} Highly virulent strains invaded the CAM, resisted phagocytosis, and proliferated. Avirulent strains were non-invasive. A well characterised avirulent strain, LOPEZ, became more virulent during intravenous or chorio-allantoic passage.²⁶ The resulting isogenic variants (LOPEZ-3 and -8) did not invade the CAM but were more successful than LOPEZ at resisting phagocytosis and surviving *in vivo*, as shown by 3.3 and 3.9 log₁₀ decreases, respectively, in LD₅₀, 72 h after intravenous inoculation.²⁶ After an intravenous dose of 10⁴ campylobacters, LOPEZ-3 exhibited a reduced initial clearance from the blood as compared with LOPEZ, and an increased ability to multiply in blood and liver.²⁶ Thus, these variants afforded a means of studying virulence determinants associated with survival apart from those related to invasion.

Cultural and morphological differences in *C. jejuni* LOPEZ and its more virulent variants LOPEZ-3 and -8

In liquid suspensions of *C. jejuni* LOPEZ prepared using growth from agar plates, the suspended cells invariably "settled out" to the bottom of the tube within 30 min. The parental strain also grew in clumps

in biphasic culture medium. In contrast, liquid suspensions of the two variants, LOPEZ-3 and -8, did not "settle out", and both grew as smooth suspensions in biphasic culture medium.

Examination of middle to late log-phase growth in biphasic medium gave further information. Gram-stained smears showed that passage *in vivo* progressively selected for cells which were thin and rod-shaped, rather than the typical spiral-shaped campylobacter cells. The percentage of thin, rod-shaped cells was 6.1, 39 and 99.9 % respectively in LOPEZ, LOPEZ-3, and LOPEZ-8; transmission electronmicroscopy showed that LOPEZ cells alone were flagellate (figs. 1A, B and C). The motility of each isolate was investigated by plating biphasic cultures in soft-agar pour plates and examining at least 1000 colonies. Only the large diffuse colonies characteristic of motile cells were seen in LOPEZ (fig. 1D). In LOPEZ-3 cultures, two colony types occurred, 99 % were small and opaque and 1 % slightly larger and opaque (fig. 1E); neither type was surrounded by the diffuse halo of growth that indicated motility. In LOPEZ-8 cultures, all colonies were small and opaque (fig. 1F).

Like the parent strain, the more virulent variants were oxidase- and catalase-positive, sensitive to nalidixic acid and resistant to cephalothin, grew at 37°C and 42°C but not at 25°C, and hydrolysed hippurate. Older cultures of the variants grown on agar plates or in biphasic medium underwent the expected gradual conversion from spiral or rod-shaped forms to a predominance of coccoid forms.

Strain LOPEZ and its two variants grew in biphasic medium at approximately the same rate between 0 and 6 h after inoculation, doubling once every 3.2 h. Between 6 and 9 h after inoculation, LOPEZ grew faster (doubling once every 1.8 h) than LOPEZ-3 and -8 (doubling times 4.3 h and 5 h, respectively). The growth rate of all three slowed from 9 to 28 h after inoculation. The final cell densities of LOPEZ-3 and -8 reached only half that of the parent strain. Therefore, the increased survival of the more virulent variants *in vivo* was not the result of a more rapid growth rate.

Cell-surface analysis of *C. jejuni* LOPEZ and its more virulent variants

This was undertaken to determine whether the increased virulence of the variants could be associated with components of the campylobacter cell envelope.

Cell-surface hydrophobicity. Because virulence in the chick embryo is associated with the ability to avoid phagocytosis,²⁶ and because cell-surface hydrophobicity influences phagocytosis,^{39–42} the relative cell-surface hydrophobicity of strain LOPEZ and its two variants was determined by hydrophobic interaction chromatography. Each organism was passed through a column of the hydrophobic gel Octyl Sepharose, and the percentage of bacteria which adsorbed to the gel was determined. The percentages for LOPEZ, LOPEZ-3 and -8 were 70.0 (SEM 3.5), 37.3 (SEM 6.3)

Fig. 1. Motility characteristics of *C. jejuni* LOPEZ, and its isogenic, more virulent variants, LOPEZ-3 and -8. Representative electronmicrographs of middle to late log-phase cells of *C. jejuni* LOPEZ (A), LOPEZ-3 (B), and -8 (C) grown in biphasic culture medium. Bar, 0.5 μ m. Appearance of *C. jejuni* LOPEZ (D), LOPEZ-3 (E), and -8 (F) in soft-agar pour plates. Each isolate was grown in biphasic culture medium and appropriate dilutions were inoculated into brucella broth containing agar 0.5%. Plates were photographed after incubation for 48 h at 37 °C.

and 28.4 (SEM 6.1) respectively, indicating that passage of strain LOPEZ through chick embryos had selected for variants with decreased cell-surface hydrophobicities. This suggested that the cell-surface hydro-

phobicity of strain LOPEZ contributed to its susceptibility to phagocytosis.²⁶ Recent studies by Tompkins⁴³ have shown that the absence of flagella does not significantly affect the cell-surface hydro-

Fig. 2. SDS-PAGE of outer-membrane proteins of *C. jejuni* LOPEZ (lane 1), LOPEZ-3 (2), and -8 (3). Mol. wt markers are shown on the left. The arrow indicates the position of the 71.4 kDa protein which is present in the profiles of *C. jejuni* LOPEZ-3 and -8, but absent from strain LOPEZ. The polypeptide band which corresponds to flagellin is indicated by the letter F.

phobicity of *C. jejuni*. Therefore, the decreased hydrophobicity of the variants probably reflected changes in the OMPs or LPS rather than loss of flagella.

Outer-membrane proteins. SDS-PAGE profiles of *C. jejuni* LOPEZ and its two variants were similar to those reported for other *C. jejuni* strains.^{12,13} The profiles are dominated by a single major OMP band (fig. 2) of 40 kDa. Other investigators have reported that a major OMP of 40–46 kDa is characteristic of *C. jejuni* and *C. coli* strains.^{2,20} A significant amount of a second protein (65 kDa) was seen in the profile of strain LOPEZ but was greatly diminished in the profiles of LOPEZ-3 and -8. Logan and Trust¹³ and Newell *et al.*¹⁸ identified proteins of similar size as flagellin. Since the variants were non-flagellate, the faint band, which migrated at approximately the same position as flagellin, may have been a co-migrating protein or flagellin in greatly reduced quantity.

One protein (71.4 kDa) was present in the profiles of LOPEZ-3 and -8 but absent from strain LOPEZ (arrow, fig. 2). Other bands, corresponding to proteins of 80.2 and 85.5 kDa, were enhanced in intensity in the profiles of the two variants. Therefore, passage of the avirulent strain LOPEZ produced more virulent isogenic variants with altered OMPs.

LPS analysis by SDS-PAGE. The LPS structures of *C. jejuni* LOPEZ and its more virulent variants were compared by analysing proteinase K-treated whole-cell lysates by SDS-PAGE and visualising the bands with silver staining. Purified LPS from the Ra and Rb chemotypes of *S. minnesota*, and proteinase K-digested whole-cell lysates from smooth, SR and the Ra chemotype of *S. typhimurium* were included as mol.-wt markers. The profiles of each isolate showed a prominent fast-migrating band, which is typically seen with a rough-type LPS (data not shown). None of the profiles appeared to have bands corresponding to

Table. The agglutination of *C. jejuni* LOPEZ and its two virulent variants by lectins

Strain	Agglutination by lectins* from	
	<i>Limax flavus</i>	<i>Helix pomatia</i>
LOPEZ	+	—
LOPEZ-3	+	wk
LOPEZ-8	+	+

—, No agglutination; +, strong agglutination; wk, weak agglutination.

* Lectin specificities according to Goldstein and Poretz,³⁶ Slifkin and Doyle,³⁷ and commercial literature of E-Y Laboratories: *Limax flavus*, N-acetylneuraminic acid > N-glycolylneuraminic acid; *Helix pomatia*, N-acetylgalactosamine α 1,3 N-acetylgalactosamine > N-acetylgalactosamine > N-acetylglucosamine > galactose.

high-mol.-wt forms of LPS, although such bands from wild-type *S. typhimurium* could be identified in the gels (data not shown). There were no apparent differences in relative migration of the LPS from LOPEZ, LOPEZ-3 or -8 and the mobility of each appeared to be identical with that of purified LPS from the Ra chemotype of *S. minnesota* and with proteinase K-digested whole-cell lysates of the Ra mutant of *S. typhimurium*.

Interaction with lectins. Passage of strain LOPEZ did not appear to select for variants with different mol.-wt forms of LPS, but this did not eliminate the possible existence of subtle structural differences in the oligosaccharide moieties. Therefore, the interaction of these organisms with various lectins was investigated. Interactions with lectins have been used to provide preliminary information about the polysaccharide components present in the cell envelopes of various gram-negative bacteria,^{36,44,45} including *C. jejuni*.^{37,46,47} *C. jejuni* LOPEZ and its two variants were unreactive with 12 of the 14 lectins tested—those derived from *Arachis hypogaeae*, *Canavalia ensiformis*, *Dolichos bifloris*, *Glycine max*, *Griffonia simplicifolia* I, *Griffonia simplicifolia* II, *Lens culinaris*, *Phaseolus vulgaris*, *Solanum tuberosum*, *Trichosanthes kinlowii*, *Triticum vulgare*, and *Ulex europaeus* I (data not shown).

All three organisms were agglutinated by the lectin derived from *Limax flavus*, indicating the presence of sialic acid on their cell surfaces (table). However, unlike the parental strain, the isogenic variants were agglutinated by the lectin from *Helix pomatia*, suggesting that terminal, non-reducing N-acetylgalactosamine groups were present on the cell surfaces of these organisms. These results indicate that passage of strain LOPEZ produced variants with modified cell-surface carbohydrates.

Discussion

Previous studies established that the virulence of *C. jejuni* for chick embryos is associated with the ability to invade the chorio-allantoic membrane, to avoid phagocytosis, and to survive and multiply *in vivo*.^{26,27}

but not with carriage of plasmid DNA, siderophore production, bacterial motility or cell-envelope toxicity.²⁷ Although this model does not closely mimic intestinal disease in mammals and does not allow an examination of early events such as colonisation, it provides a useful system for the study of invasion, resistance to phagocytosis and growth *in vivo*. The present studies focused on the cultural, morphological and cell-surface properties of a well characterised avirulent non-invasive *C. jejuni* strain and two isogenic variants obtained by passage *in vivo* to determine whether virulence could be associated with one or more components of the campylobacter cell envelope.

Motility was shown previously to play little role in the ability of invasive *C. jejuni* to cross the CAM of chick embryos and survive *in vivo*. These results confirm that flagella are not required for the survival of campylobacters in the chick embryo.²² Aflagellate forms progressively increased when *C. jejuni* LOPEZ was passaged intravenously through embryos. The isogenic variant LOPEZ-3 was non-motile in soft-agar pour plates and non-flagellate as determined by electronmicroscopy. When *C. jejuni* LOPEZ-3 was subjected to additional chorio-allantoic passages, the phenotype of the resulting variant (LOPEZ-8) remained non-motile. Therefore, flagella are unnecessary for growth in the chick embryo.

In a recent review, Smith⁴⁸ pointed out that the behaviour of micro-organisms *in vivo* is determined both by genetic make-up and environment. Selection and phenotypic change may occur when pathogens are moved from one type of environment to another. Selection for non-motile forms *in vivo* is not without precedent. Luzar and colleagues⁴⁹ reported that strains of *Pseudomonas aeruginosa* isolated from the lungs of patients with cystic fibrosis are frequently non-motile and non-chemotactic. These authors suggest that after initial pulmonary invasion, there is progressive host selection against bacterial properties normally associated with invasion, such as motility and chemotaxis. The present study suggests that such a selective pressure occurs in the chick embryo.

These findings do not negate the importance of motility in the viscous environment of the gastrointestinal tract where both the spiral shape and propulsion by flagella give campylobacter cells a selective advantage.^{3-5, 50} However, there is little evidence to support a role for this cell-surface structure in invasion or extra-intestinal survival and proliferation.

In addition to the loss of flagella, other cell-surface changes were associated with the enhanced virulence of the two passaged variants, including alteration in cultural characteristics, cellular morphology, cell-surface hydrophobicity, OMPs and cell-surface carbohydrates. It is difficult to assess which of these changes directly contributed to the enhanced survival of *C. jejuni* LOPEZ-3 and -8 *in vivo*. However, since surface hydrophobicity facilitates phagocytosis,^{39, 40, 41, 42} it is likely that the decreased cell-surface hydrophobicity of

the variants contributed to their ability to avoid phagocytosis.

Changes in the OMP or LPS composition of the variants may have contributed to their decreased cell-surface hydrophobicity. At least one new protein (71.4 kDa) was expressed in the outer membrane of LOPEZ-3 and -8. Two additional OMPs (80.2 and 85.5 kDa) present in strain LOPEZ were found in greater amounts in the passaged variants. One or more of these proteins may have hydrophilic properties which help in preventing phagocytosis.

In-vivo passage of *C. jejuni* LOPEZ did not appear to have selected isogenic variants with LPS of different mol. wt, but subtle alterations in LPS structure may nonetheless have occurred. Indeed, the observation that the two variants reacted with a lectin with which the parental strain was unreactive suggests an alteration in cell-surface carbohydrates. Recent studies have demonstrated that sialic acid is linked to *C. jejuni* LPS.^{21, 22} Sialylation of gonococcal LPS *in vivo* by a host substrate, CMP-N-acetylneuraminic acid, has been shown to confer serum resistance on this pathogen.⁴⁸ While sialic acid was detected on the cell surface of *C. jejuni* LOPEZ and its variants, it was the presence of N-acetylgalactosamine on the surface of the latter which seemed to correlate with virulence *in vivo*. A more complete chemical analysis of the LPS of the organisms is required to confirm this finding.

The significance of the morphological alterations which occurred when strain LOPEZ was passaged through the embryos is unclear. Certainly, *C. jejuni* is a pleomorphic micro-organism, which is known to undergo a morphological change from curved or spiral forms in young cultures to coccoid forms in older cultures.^{51, 52} However, in-vivo selection of rod-shaped cells from spiral forms had not previously been reported. It is unlikely that this morphological alteration was related to the loss of flagella, since loss of flagella either in natural variants or in FlaA or FlaB mutants does not result in rod-shaped cells.^{53, 54} When non-motile clones of some *C. jejuni* strains are repeatedly subcultured in soft agar, rod-shaped cells sometime arise. However, the reason that non-flagellate rod-shaped cells (LOPEZ-8) were progressively selected *in vivo* from a mixed population containing both non-flagellate rod-shaped and non-flagellate spiral cells (LOPEZ-3) remains to be elucidated. Presumably, cells with the altered cellular morphology had a selective advantage in the embryonic host.

The virulence of *C. jejuni* in the chick embryo appears to be relatively complex. Successful multiplication *in vivo* is clearly associated with the ability to resist phagocytosis. Several cell-surface changes may contribute to the enhanced virulence of variants produced by passage through chick embryos, including alterations in cellular morphology, decreases in cell-surface hydrophobicity, changes in the OMPs and alterations in cell-surface carbohydrates.

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References

- Cover TL, Blaser MJ. The pathobiology of *Campylobacter* infections in humans. *Annu Rev Med* 1989; **40**: 269–285.
- Walker RI, Caldwell MB, Lee EC, Guerry P, Trust TJ, Ruiz-Palacios GM. Pathophysiology of *Campylobacter* enteritis. *Microbiol Rev* 1986; **50**: 81–94.
- Lee A, O'Rourke JL, Barrington PJ, Trust TJ. Mucus colonization as a determinant of pathogenicity in intestinal infection by *Campylobacter jejuni*: a mouse cecal model. *Infect Immun* 1986; **51**: 536–546.
- Morooka T, Umeda A, Amako K. Motility as an intestinal colonization factor for *Campylobacter jejuni*. *J Gen Microbiol* 1985; **131**: 1973–1980.
- Aguero-Rosenfeld ME, Yang X-H, Nachamkin I. Infection of adult Syrian hamsters with flagellar variants of *Campylobacter jejuni*. *Infect Immun* 1990; **58**: 2214–2219.
- McSweeney E, Walker RI. Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. *Infect Immun* 1986; **53**: 141–148.
- Newell DG, McBride H, Dolby JM. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. *J Hyg* 1985; **95**: 217–227.
- Caldwell MB, Guerry P, Lee EC, Burans JP, Walker RI. Reversible expression of flagella in *Campylobacter jejuni*. *Infect Immun* 1985; **50**: 941–943.
- Harris LA, Logan SM, Guerry P, Trust TJ. Antigenic variation of *Campylobacter* flagella. *J Bacteriol* 1987; **169**: 5066–5071.
- Buchanan TM, Pearce WA. Pathogenic aspects of outer membrane components of gram-negative bacteria. In: Inouye M (ed) *Bacterial outer membranes: biogenesis and functions*. New York, John Wiley and Sons. 1979: 475–514.
- Beer W, Adam M, Seltmann G. Monosaccharide composition of lipopolysaccharides from *Campylobacter jejuni* and *Campylobacter coli*. *J Basic Microbiol* 1986; **26**: 201–204.
- Blaser MJ, Hopkins JA, Berka RM, Vasil ML, Wang W-L L. Identification and characterization of *Campylobacter jejuni* outer membrane proteins. *Infect Immun* 1983; **42**: 276–284.
- Logan SM, Trust TJ. Outer membrane characteristics of *Campylobacter jejuni*. *Infect Immun* 1982; **38**: 898–906.
- Logan SM, Trust TJ. Structural and antigenic heterogeneity of lipopolysaccharides of *Campylobacter jejuni* and *Campylobacter coli*. *Infect Immun* 1984; **45**: 210–216.
- Naess V, Hofstad T. Isolation and chemical composition of lipopolysaccharide from *Campylobacter jejuni*. *APMIS* 1982; **90**: 135–139.
- Naess V, Hofstad T. Chemical composition and biological activity of lipopolysaccharides prepared from type strains of *Campylobacter jejuni* and *Campylobacter coli*. *APMIS* 1984; **92**: 217–222.
- Naess V, Hofstad T. Chemical studies of partially hydrolyzed lipopolysaccharides from four strains of *Campylobacter jejuni* and two strains of *Campylobacter coli*. *J Gen Microbiol* 1984; **130**: 2783–2789.
- Newell DG, McBride H, Pearson AD. The identification of outer membrane proteins and flagella of *Campylobacter jejuni*. *J Gen Microbiol* 1984; **130**: 1201–1208.
- Perez Perez GI, Blaser MJ. Lipopolysaccharide characteristics of pathogenic campylobacters. *Infect Immun* 1985; **47**: 353–359.
- Trust TJ, Logan SM. Outer membrane and surface structure of *Campylobacter jejuni*. In: Butzler JP (ed) *Campylobacter infection in man and animals*. Boca Raton, FL, CRC Press, Inc. 1984; 133–142.
- Moran AP, Rietschel ET, Kosunen TU, Zähringer U. Chemical characterization of *Campylobacter jejuni* lipopolysaccharides containing N-acetylneuraminic acid and 2,3-diamino-2,3-dideoxy-D-glucose. *J Bacteriol* 1991; **173**: 618–626.
- Mills SD, Aspinall GO, McDonald AG, Raju TS, Kurjanczyk LA, Penner JL. Lipopolysaccharide antigens of *Campylobacter jejuni*. In: Nachamkin I, Blaser MJ, Tompkins LS (eds) *Campylobacter jejuni—current status and future trends*. Washington, DC. American Society for Microbiology 1992: 232–229.
- Pei Z, Ellison RT, Blaser MJ. Identification, purification, and characterization of major antigenic proteins of *Campylobacter jejuni*. *J Biol Chem* 1991; **266**: 16363–16369.
- De Melo MA, Pechère J-C. Identification of *Campylobacter jejuni* surface proteins that bind to eucaryotic cells in vitro. *Infect Immun* 1990; **58**: 1749–1756.
- Fauchère JL, Kervella M, Roseanu A, Pages JM, Fendri C. In vitro study of virulence factors of enteric *Campylobacter* spp. In: Nachamkin I, Blaser MJ, Tompkins LS (eds) *Campylobacter jejuni—current status and future trends*. Washington, DC. American Society for Microbiology 1992: 168–175.
- Field LH, Underwood JL, Payne SM, Berry LJ. Virulence of *Campylobacter jejuni* for chicken embryos is associated with decreased bloodstream clearance and resistance to phagocytosis. *Infect Immun* 1991; **59**: 1448–1456.
- Field LH, Headley VL, Underwood JL, Payne SM, Berry LJ. The chicken embryo as a model for *Campylobacter* invasion: comparative virulence of human isolates of *Campylobacter jejuni* and *Campylobacter coli*. *Infect Immun* 1986; **54**: 118–125.
- Smyth CJ, Jonsson P, Olsson E *et al.* Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infect Immun* 1978; **22**: 462–472.
- Field LH, Headley VL, Payne SM, Berry LJ. Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. *Infect Immun* 1986; **54**: 126–132.
- Inouye M, Guthrie JP. A mutation which changes a membrane protein of *E. coli*. *Proc Natl Acad Sci USA* 1969; **64**: 957–961.
- Filip C, Fletcher G, Wulff JL, Earhart CF. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol* 1973; **115**: 717–722.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
- Ames GF-L. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. Membrane, soluble and periplasmic fractions. *J Biol Chem* 1974; **249**: 634–644.
- Kimura A, Hansen EJ. Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. *Infect Immun* 1986; **51**: 69–79.
- Tsai C-M. The analysis of lipopolysaccharide (endotoxin) in meningococcal polysaccharide vaccines by silver staining following SDS-polyacrylamide gel electrophoresis. *J Biol Stand* 1986; **14**: 25–33.
- Goldstein IJ, Poretz RD. Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins. In: Liener IE, Sharon N, Goldstein IJ (eds) *The lectins—properties, functions, and applications in biology and medicine*. New York, Academic Press, Inc. 1986: 33–247.
- Slifkin M, Doyle RJ. Lectins and their application to clinical microbiology. *Clin Microbiol Rev* 1990; **3**: 197–218.
- Wong KH, Skelton SK, Feeley JC. Strain characterization and grouping of *Campylobacter jejuni* and *Campylobacter coli* by interaction with lectins. *J Clin Microbiol* 1986; **23**: 407–410.
- van Oss CJ. Phagocytosis as a surface phenomenon. *Annu Rev Microbiol* 1978; **32**: 19–40.
- van Oss CJ, Gillman CF, Neumann AW. Phagocytic engulfment and cell adhesiveness. New York, Marcel Dekker, Inc. 1975.
- Ofek I, Rest RF, Sharon N. Nonopsonic phagocytosis of microorganisms. *ASM News* 1992; **58**: 429–435.
- Ohman L, Maluszynska G, Magnusson K-E, Stendahl O. Surface interaction between bacteria and phagocytic cells. *Prog Drug Res* 1988; **32**: 131–147.
- Tompkins LS. Genetic and molecular approaches to *Campylo-*

- bacter pathogenesis. In: Nachamkin I, Blaser MJ, Tompkins LS (eds) *Campylobacter jejuni*—current status and future trends. Washington, DC. American Society for Microbiology 1992: 241–254.
44. Lis H, Sharon N. Lectins as molecules and tools. *Annu Rev Biochem* 1986; **55**: 35–67.
45. Pistole TG. Interaction of bacteria and fungi with lectins and lectin-like substances. *Annu Rev Microbiol* 1981; **35**: 85–112.
46. Corbel MJ, Gill KPW. Lectin agglutination of thermophilic *Campylobacter* species. *Vet Microbiol* 1987; **15**: 163–173.
47. Wong KH, Skelton SK, Feeley JC. Interaction of *Campylobacter jejuni* and *Campylobacter coli* with lectins and blood group antibodies. *J Clin Microbiol* 1985; **22**: 134–135.
48. Smith H. Pathogenicity and the microbe *in vivo*. *J Gen Microbiol* 1990; **136**: 377–383.
49. Luzar MA, Thomassen MJ, Montie TC. Flagella and motility alterations in *Pseudomonas aeruginosa* strains from patients with cystic fibrosis: relationship to patient clinical condition. *Infect Immun* 1985; **50**: 577–582.
50. Ferrero RL, Lee A. Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. *J Gen Microbiol* 1988; **134**: 53–59.
51. Karmali MA, Skirrow MB. Taxonomy of the genus *Campylobacter*. In: Butzler JP (ed) *Campylobacter* infection in man and animals. Boca Raton, FL, CRC Press, Inc. 1984: 1–20.
52. Smibert RM. Genus *Campylobacter* (Sebald and Veron, 1963). In: Krieg NR (ed) *Bergey's manual of systematic bacteriology*, vol. 1. Baltimore, The Williams and Wilkins Co. 1984: 111–118.
53. Nachamkin I, Yang X-H. Immune response to *Campylobacter* flagellin. In: Nachamkin I, Blaser MJ, Tompkins LS (eds) *Campylobacter jejuni*—current status and future trends. Washington, DC. American Society for Microbiology. 1992: 216–222.
54. Guerry P, Alm RA, Power ME, Trust T. Molecular and structural analysis of *Campylobacter* flagellin. In: Nachamkin I, Blaser MJ, Tompkins LS (eds) *Campylobacter jejuni*—current status and future trends. Washington, DC. American Society for Microbiology. 1992: 267–281.