

# Expression patterns and role of the CadF protein in *Campylobacter jejuni* and *Campylobacter coli*

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## Keywords

adhesion; *Campylobacter*; CadF; fibronectin; invasion; pathogenesis.

## Abstract

Binding of *Campylobacter jejuni* and *Campylobacter coli* to host fibronectin is mediated by the 37 kDa outer membrane protein CadF. Immunoblot analysis of 58 *C. jejuni* and *C. coli* isolates of human and animal origin showed that CadF is expressed in every strain. In most *C. jejuni* isolates, a 37 kDa band (p37) and a less-prominent 32 kDa band (p32) reacted with the antibodies. In *C. coli* isolates, CadF was consistently larger with sizes of 39 kDa (p39) and 34 kDa (p34), respectively. PCR analysis and sequencing revealed the presence of a 39-bp insertion sequence in the *cadF* gene of *C. coli* strains, explaining the increased molecular size. Infection assays revealed that *C. jejuni* bound and invaded INT-407 epithelial cells much more efficiently than *C. coli* and that this difference was considerably reduced in isogenic *cadF* mutants. These results demonstrate that CadF is an important pathogenicity factor. The difference between CadF of *C. jejuni* and *C. coli* may potentially be exploited to discriminate these species in food and clinical specimens.

## Introduction

*Campylobacter jejuni* and *Campylobacter coli* are major causes of gastrointestinal diseases worldwide (Altekruse *et al.*, 1999; Akitoeye *et al.*, 2002). These pathogens colonize and invade the intestinal mucosa *in vitro* (Hu & Kopecko, 1999; Bacon *et al.*, 2000; Biswas *et al.*, 2000; Monteville *et al.*, 2003; Nadeau *et al.*, 2003; Konkel *et al.*, 2004; Hu *et al.*, 2005). *Campylobacter jejuni* synthesizes a set of proteins called *Campylobacter* invasion antigens (Cia proteins) that may contribute to the invasion of epithelial cells (Konkel *et al.*, 1999a). *Campylobacter jejuni* also possesses a 37 kDa adhesin, termed CadF, that binds fibronectin and aids the adherence of *C. jejuni* to intestinal epithelial cells (Konkel *et al.*, 1997, 1999b, 2005). *CadF* is a single-copy, highly conserved chromosomal gene of *Campylobacter* (Konkel *et al.*, 1999b; Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006). Using an overlapping peptide library derived from CadF, maximal fibronectin-binding activity was localized within 4 amino acids (aa) (134–137 aa) con-

sisting of the phenylalanine–arginine–leucine–serine motif (Konkel *et al.*, 2005). Previous work based on immunoblot analysis of clinical isolates indicated that the CadF protein is highly conserved among *C. jejuni* strains from the US (Konkel *et al.*, 1997, 1999b). Therefore, a variety of assays could be developed based on the detection of the *cadF* virulence gene and its product. In the present study, the CadF proteins of a large number of *C. jejuni* and *C. coli* strains of human and animal origin were compared, and the role of CadF in the attachment and internalization of INT-407 epithelial cells was determined.

## Materials and methods

### *Campylobacter* wild-type strains and growth conditions

Table 1 shows the collection of isolates used in this study. Bacteria were grown (48 h) on *Campylobacter* blood-free selective agar base with growth supplement at 37 °C under

**Table 1.** *Campylobacter* isolates used in the study and detection of CadF proteins

Species	Origin	Strain designation	Presence of CadF protein bands		
			37 kDa (p37)	32 kDa (p32)	
<i>C. jejuni</i>	Human, feces	ATCC 43431	+	+	
		NCTC 11168	+	+	
		81-176	+	+	
		1543/01	+	+	
		ST3046	+	+	
		81116	+	+	
		F38011	+	+	
		CDC 2004-341	+	+	
		158/96	+	-	
		157/96	+	-	
		51/89	+	-	
		230205ZH0017	+	-	
		230205ZH0018	+	-	
		Chicken, intestine	G 447	+	+
			G 448	+	+
	G 450		+	+	
	G 451		+	+	
	G 464		+	+	
	G 465		+	+	
	G 467		+	+	
	G 477		+	+	
	G 478		+	+	
	G 479		+	+	
	Chicken, cloaca	RM1849	+	+	
		RM1221	+	+	
		151003ZH0099	+	+	
		1991	+	+	
		201004ZH0078	+	+	
		503	+	+	
		av245	+	+	
		ALK 1116	+	+	
		ATCC 43430	+	+	
		C 130	+	-	
Calf, abomasum	73 Di	+	+		
	100204ZH0021	+	+		
<i>C. coli</i> *	Pig, feces	ALK 1158	+	+	
		ALK 1179	+	-	
		ALK 1184	+	+	
		ALK 1185	+	-	
		ALK 1187	+	+	
		ALK 1290	+	-	
		ALK 1295	+	+	
		ALK 1233	+	+	
		ALK 1282	+	-	

**Table 1.** Continued.

Species	Origin	Strain designation	Presence of CadF protein bands	
			37 kDa (p37)	32 kDa (p32)
	Chicken, intestine	G 427	+	+
		G 472	+	+
	Poultry, feces	Han35	+	+
		Han36	+	+
		Han135	+	+
		2371	+	+
	Poultry, liver	K1102/03	+	+
	Quail, intestine	G 510	+	-
	Turkey	av352	+	-

\*In these strains the CadF protein is slightly larger (39 and 34 kDa, respectively).

microaerophilic conditions generated by CampyGen (Oxoid, Basingstoke, UK). Species identification was based on biochemical tests (catalase, oxidase, urease activity, hippurate and indoxyl acetate hydrolysis, and sensitivity to cephalothin and nalidixic acid) and a multiplex PCR assay (Cloak & Fratamico, 2002; Oyarzabal *et al.*, 2005).

#### PCR and analysis of amplified products

The *cadF* gene and its flanking regions were amplified by PCR using the following primers. CadF1 Fwd: 5'-TTG CTC TAA AGG ATA ACC TAT GA-3', CadF1 Rev: 5'-TAT GGA CGC CGC AAA GCA AG-3', CadF2 Fwd: 5'-CCA CTC TTC TAT TAT CCG CTC TAC C-3', and CadF2 Rev: 5'-GGT GCT GAT AAC AAT GTA AAA TTT G-3'. PCR conditions were as follows: denaturation (94 °C, 2 min), six cycles of touchdown PCR (94 °C for 30 s, 58 °C for 45 s, decreasing 0.5 °C per cycle, 72 °C for 2 min), followed by 30 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 2 min and a final extension step at 72 °C for 10 min. Amplified products were analyzed by agarose gel electrophoresis, cloned into pGEM-T-easy vector (Promega, Madison) and sequenced. Nucleotide sequence analysis and protein sequence alignments were performed using free software (<http://searchlauncher.bcm.tmc.edu/seq-util/Options/sixframe.html>; <http://www.ebi.ac.uk/clustalw>).

#### Generation of *cadF* mutants and growth conditions

The *cadF* gene and its flanking regions from *C. jejuni* 81116 were amplified by PCR using the primers CadF3 Fwd: 5'-GAT AAA GCA TTC TAA ACA TT-3' and CadF3 Rev: 5'-GAG CAC CCA CAC ACT GCA C-3'. The fragment was ligated into pGEM-T-easy vector and transformed into *Escherichia coli* JM110. An inactivated *cadF* of strain 81116 was obtained by insertion of the Aph-A3 kanamycin

resistance cassette (1.5 kb) at the BclI site and introduced into the 81116 genome by homologous recombination. The *cadF* mutant in *C. jejuni* F38011 was generated as described (Konkel *et al.*, 1997). Disruption of the *cadF* gene in each strain was confirmed by PCR. The *cadF* mutant strains 81116 $\Delta$ *cadF* and F38011 $\Delta$ *cadF* were grown on Columbia agar with 5% blood and 20  $\mu$ g mL<sup>-1</sup> kanamycin, and on Mueller–Hinton (MH) agar amended with 20  $\mu$ g mL<sup>-1</sup> kanamycin, respectively.

### Infection of INT-407 cells

Human embryonic intestinal epithelial cells (INT-407, ATCC-CCL-6) were grown in Eagle's minimum essential medium (MEM) containing L-glutamine and Earle's salts (Invitrogen), 100 U mL<sup>-1</sup> penicillin, 100  $\mu$ g mL<sup>-1</sup> streptomycin and 10% fetal bovine serum (FBS, Invitrogen) in a humidified 5% CO<sub>2</sub> incubator. For infection assays, cells were grown (48 h) in 12-well tissue culture plates to reach ~70% confluence. Then, the medium was replaced with MEM without antibiotics and bacteria were added at a multiplicity of infection (MOI) of 100. After 6 h of incubation, the cells were washed three times with 1 mL of medium and suspended, diluted and plated on MH agar plates to determine the total number of cell-associated bacteria (attached and intracellular), or incubated with gentamicin (250  $\mu$ g mL<sup>-1</sup>, 2 h) to kill all extracellular bacteria, and then disrupted with saponin (0.1%, 37 °C, 15 min). Released intracellular bacteria were diluted and plated as described above. The level of total cell-associated and intracellular bacteria was determined by calculating the number of CFU. All experiments were performed in triplicate.

### Generation of the polyclonal CadF antibodies

Polyclonal antiserum ( $\alpha$ -CadF-1) was raised according to standard protocols (Biogenes, Berlin, Germany) by immunization of two rabbits with a conserved *C. jejuni* CadF-derived peptide (293–306 aa: QDNPRSSNDTKEGR) conjugated to Limulus polyphemus hemocyanin carrier protein. Immunoblot analysis verified that  $\alpha$ -CadF-1 is specific for *C. jejuni* CadF and does not react with CadF from *C. coli*. The polyclonal rabbit antiserum  $\alpha$ -CadF-2 was obtained by immunization with gel-purified CadF and reacts with both CadF from *C. coli* and *C. jejuni* (Konkel *et al.*, 1997).

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Whole bacterial cells harvested from agar plates or infected INT-407 cells were lysed in SDS-PAGE buffer (2% SDS, 0.1 M dithiothreitol), boiled, separated on 12% polyacryla-

mid gels and either stained with Coomassie–Brilliant Blue or blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The blots were incubated with the polyclonal antibodies or with a monoclonal  $\alpha$ -GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz) and, subsequently, with horseradish peroxidase-conjugated  $\alpha$ -rabbit IgG or  $\alpha$ -goat IgG (Dako, Hamburg, Germany). Immuno-reactive bands were visualized with ECL plus a Western Blotting Detection System (Amersham-Bioscience).

### Statistical analysis

All data were analyzed using the Student *t*-test with SIGMA-STAT statistical software (version 2.0), with significance set at  $P \leq 0.01$  (\*) and  $P \leq 0.001$  (\*\*).

## Results

### Immunodetection of CadF in *C. jejuni* isolates

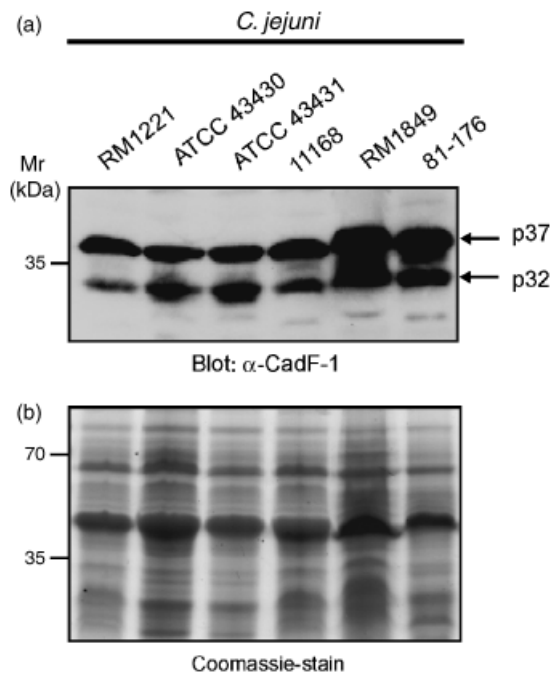
The 58 *Campylobacter* isolates used in this study were characterized as *C. jejuni* (40 strains) and *C. coli* (18 strains). The *C. jejuni* isolates included strains isolated from both humans and animals, while all the *C. coli* strains were recovered from animals (Table 1).

Using two CadF-specific antisera, a 37 kDa band (p37) and a less-prominent 32 kDa band (p32) were detected in *C. jejuni* strains by immunoblotting of total cell lysates. These bands corresponded to previously described CadF proteins (Konkel *et al.*, 1997; Mamelli *et al.*, 2006, 2007). While p37 was present in all *C. jejuni* isolates, five human isolates and one from a calf failed to exhibit the less-prominent p32 band (Table 1). A representative gel and immunoblot with  $\alpha$ -CadF-1 of several *C. jejuni* isolates are shown in Fig. 1a. Equivalent amounts of proteins present were confirmed by Coomassie staining for all tested strains (Fig. 1b).

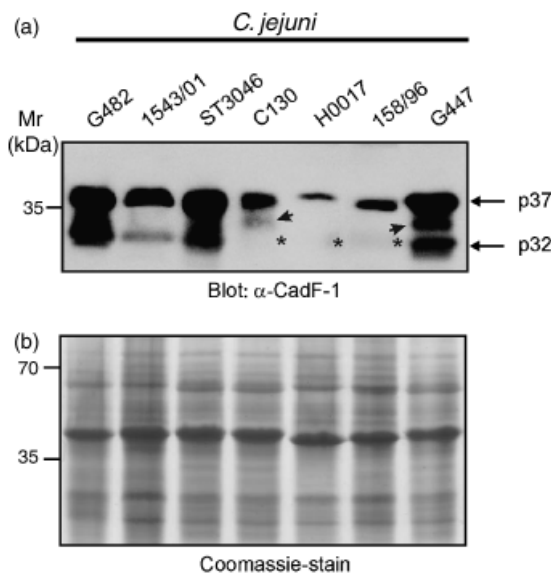
To verify the specificity of the  $\alpha$ -CadF antibodies, two isogenic *cadF* mutants were produced in strains 81116 and F38011. These mutants lacked the p37 and p32 bands observed for the parent strain (Fig. 5a, arrows). As expected, whole-cell extracts of *Campylobacter fetus*, *Helicobacter pylori* or *E. coli* controls did not react with the CadF-specific antisera (data not shown).

### Variability of CadF proteins among *C. jejuni* and *C. coli* isolates

Although the pattern of  $\alpha$ -CadF-1 antibody reactivity was largely identical among the isolates, the number and intensities of the CadF protein species slightly varied among *C. jejuni* strains (Fig. 2a, arrows and asterisks), despite loading equivalent amounts of proteins (Fig. 2b). In some



**Fig. 1.** Representative immunoblot analysis of total bacterial cell lysates showing CadF immunoreactivity with the  $\alpha$ -CadF-1 antibody among *Campylobacter jejuni* isolates. (a) *Campylobacter jejuni* isolates showing 32 and 37 kDa bands corresponding to the CadF proteins. (b) Coomassie staining showing equivalent amounts of protein (50  $\mu$ g) in each lane.



**Fig. 2.** Variability in number and band intensity of CadF proteins in *Campylobacter jejuni*. (a) Immunoblot analysis indicating the variability in  $\alpha$ -CadF-1 staining among strains. Arrows indicate additional bands in the pattern and asterisks indicate bands that are absent in some strains. (b) Coomassie staining showing equivalent amounts of protein (50  $\mu$ g) in each lane.

cases, intermediate CadF bands of  $\sim$ 34 kDa were also observed (Fig. 2a, arrows).

Interestingly, in all *C. coli* isolates tested, CadF was slightly larger and had a weaker expression, as judged from Western blot analysis with  $\alpha$ -CadF-1 antibody (Fig. 3a). All *C. coli* isolates exhibited a 39 kDa band (p39), while a lower migrating 34 kDa band (p34) was detected in 12 out of 18 *C. coli* strains (Fig. 3a, Table 1).

### PCR amplification and sequencing of *cadF* genes

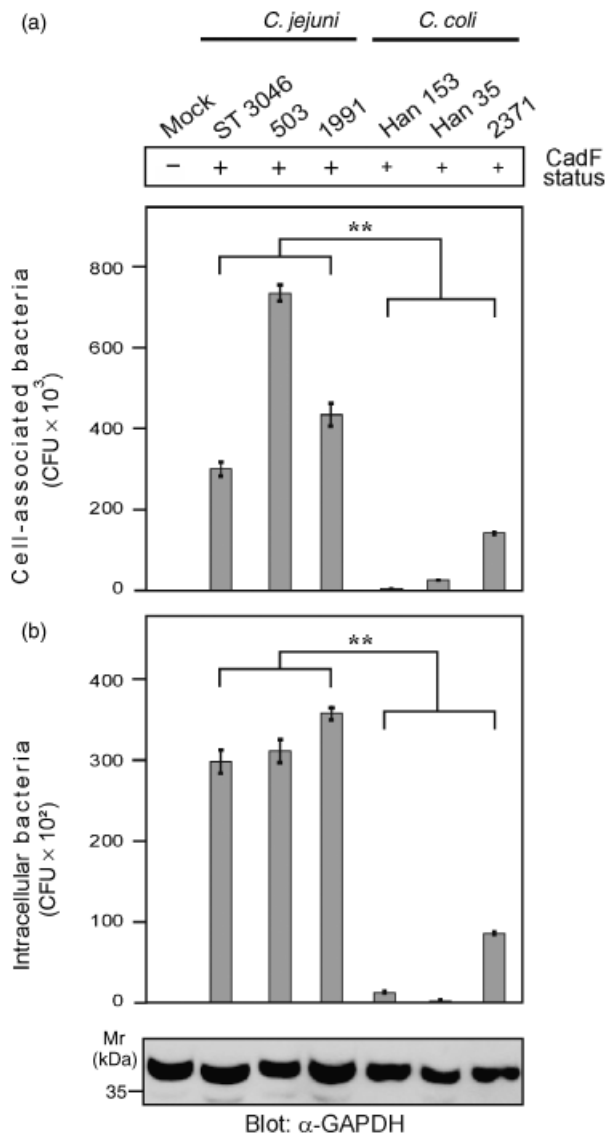
To elucidate the differences in CadF protein size and expression between *C. jejuni* and *C. coli* strains, sequence analyses on a set of *cadF* genes were performed. PCR analysis of the *C. coli* strains revealed a slightly larger *cadF* than that of *C. jejuni* 81116 (1320 vs. 1285 bp for *cadF* and some flanking sequence, respectively) (Fig. 3b, arrows). A different PCR with primers directed against the most conserved parts within the *cadF* gene yielded 930 bp for *C. coli* strains and 890 bp for *C. jejuni* 81116 (Fig. 3b, arrows). Insertion of a kanamycin resistance cassette in 81116 $\Delta$ *cadF* mutant resulted in a 1.5 kb increase in product size in both PCRs, as expected (Fig. 3b, arrowheads).

Sequencing of the *cadF* coding region from three *C. coli* isolates consistently revealed an additional sequence (39 bp) at the indicated position compared with *cadF* of *C. jejuni* (Fig. 3c). Analysis of the *cadF* sequences from three *C. jejuni* and one *C. coli* available sequenced genomes (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006) confirmed the findings of this study. Alignment of deduced amino acid sequences showed that the CadF protein from *C. coli* strains is 13 aa larger than those from *C. jejuni* (Fig. 3d), in agreement with the size differences seen in the Western blots (Fig. 3a).

### Binding and invasion of INT-407 cells by differently CadF-expressing *C. jejuni* and *C. coli* strains

Possible differences in bacterial adhesion and invasion between the CadF-expressing *C. jejuni* and *C. coli* isolates were explored in infection assays with INT-407 cells. Quantification of cell-associated (Fig. 4a) and intracellular bacteria (Fig. 4b) by the gentamicin protection assay revealed that the *C. jejuni* isolates expressing p37 CadF exhibited significantly higher binding and invasion rates than *C. coli* strains expressing p39 CadF ( $P \leq 0.001$ ). The *C. coli* isolates Han35 and Han153 exhibited the lowest values of cell-associated and intracellular bacteria. To determine the overall contribution of the CadF protein in the binding and invasion of *C. jejuni* to INT-407 cells, the interactions of *C. jejuni* 81116 $\Delta$ *cadF* and F38011 $\Delta$ *cadF* mutant strains with cells were examined. Immunoblot analysis with  $\alpha$ -CadF-1 confirmed that the CadF protein was not synthesized by either *cadF* mutant strain (Fig. 5a). Significant reduction ( $\sim$ 50%)



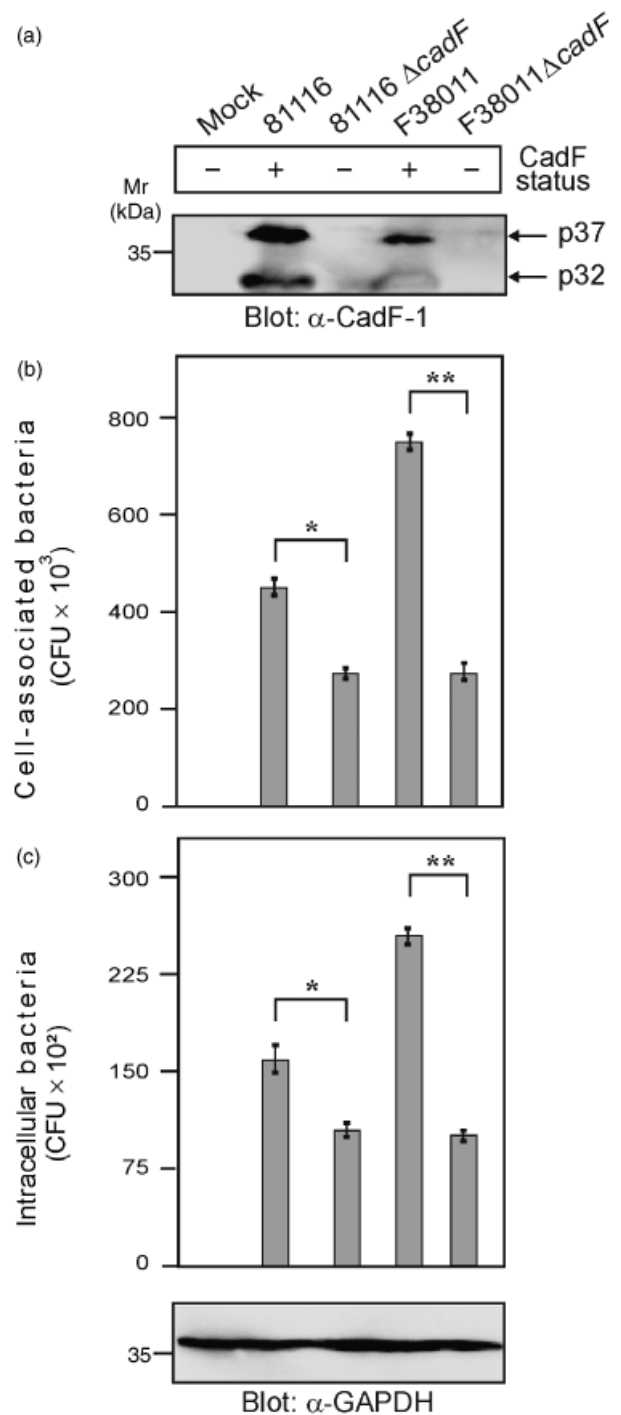


**Fig. 4.** Effect of CadF on adhesion to and invasion of INT-407 cells with *Campylobacter jejuni* and *Campylobacter coli* isolates. (a) Total and (b) intracellular *Campylobacter* cells were quantified by gentamicin protection assay. \*\* Statistically significant ( $P \leq 0.001$ ).  $\alpha$ -GAPDH staining was used as loading control.

in adherence and invasion was observed for the *C. jejuni* 81116 $\Delta$ cadF and F38011 $\Delta$ cadF mutant strains (Fig. 5b and c). These findings demonstrate that CadF is an important pathogenicity factor.

## Discussion

The pathogenicity of several *Campylobacter* species is dependent on their ability to attach and invade the human intestine. One of the adhesion factors that *C. jejuni* uses to attach, and eventually to invade mammalian cells, is CadF, a



**Fig. 5.** Effect of CadF expression on adhesion and invasion of *Campylobacter jejuni*. INT-407 cells were infected for 6 h at 37 °C with wild-type 81116 vs. 81116 $\Delta$ cadF and wild-type F38011 vs. F38011 $\Delta$ cadF. (a) The expression of CadF proteins during infection was verified by immunoblotting using the  $\alpha$ -CadF-1 antibody. (b) Total and (c) intracellular *Campylobacter* cells were quantified by gentamicin protection assay. (\*\*\*) Statistically significant ( $P \leq 0.001$ ,  $P \leq 0.01$ ).  $\alpha$ -GAPDH staining was used as loading control.

protein that binds to fibronectin – a component of the extracellular matrix (Konkel *et al.*, 1997). The importance of CadF for the binding of *C. jejuni* to epithelial cells has been demonstrated *in vitro* (Konkel *et al.*, 1997) and *in vivo* (Ziprin *et al.*, 1999). The primary aim of this study was to determine the genetic and functional diversity of CadF protein among a large number of *C. jejuni* and *C. coli* isolates.

Western blotting analyses with two highly specific  $\alpha$ -CadF antibodies showed a prominent 37 kDa CadF protein (p37) as well as a less-prominent 32 kDa (p32) protein for all tested *C. jejuni* isolates. Both bands were absent in two isogenic *cadF* knockout mutants. The results, which are consistent and extend earlier observations (Konkel *et al.*, 1997, 1999b), also revealed that the number and intensity of CadF bands varied among *C. jejuni* strains. While p37 was detected in all *C. jejuni* isolates of human and animal origin, the less-prominent p32 band was found only in 62% of the *C. jejuni* isolates of human origin and in 96% of the *C. jejuni* isolates of animal origin. Heat modifiability is a well-known feature of membrane proteins (Nakamura & Mizushima, 1976; Bolla *et al.*, 1995), including CadF (Konkel *et al.*, 1997, 1999b; Mamelli *et al.*, 2006, 2007). Therefore, the migration of CadF as two protein species is likely caused by their heat-modifiable conformational state, where p32 is the incompletely denatured and partially folded form of CadF.

In contrast to earlier reports, where the CadF protein was found to be conserved in size and antigenicity among *C. jejuni* and *C. coli* isolates from US (Konkel *et al.*, 1999b), it was observed that all *C. coli* isolates tested possessed a larger CadF (p39 and p34) than *C. jejuni*. Sequence analysis of three *C. coli* isolates confirmed this difference between species and indicated that *C. coli* carried a stretch of 13 aa in the middle region of the protein. Interestingly, the latter insertion sequence was not found in one *C. coli* isolate from the US, which instead contained another insertion sequence of 7 aa (Konkel *et al.*, 1999b). However, whether the differences in amino acid sequence or a lower expression level accounted for the apparent weaker immunoreactivity of the *C. coli* CadF with the polyclonal antisera of this study remains to be determined. Nevertheless, data of this study strongly suggest that the differences in molecular size and differences in nucleotide sequence between the *C. jejuni* and *C. coli* isolates may be a suitable diagnostic marker to discriminate between these species in food and clinical specimen.

The possible biological significance of the variation in CadF was investigated by comparing a subset of *C. jejuni* and *C. coli* strains for their ability to infect INT-407 intestinal epithelial cells, which serves as an *in vitro* model system for *C. jejuni* and *C. coli* attachment and invasion (Hu & Kopecko, 1999; Bacon *et al.*, 2000; Biswas *et al.*, 2000; Monteville *et al.*, 2003; Nadeau *et al.*, 2003; Konkel *et al.*,

2004; Hu *et al.*, 2005). Interestingly, *C. jejuni* strains adhered and invaded INT-407 cells at significantly greater levels than *C. coli* strains. This effect was at least in part caused by CadF as the 81116 $\Delta$ *cadF* and F38011 $\Delta$ *cadF* mutants showed reduced adhesion, which is consistent with previous studies showing a reduced adherence to INT-407 cells of a *C. jejuni cadF* mutant (Konkel *et al.*, 1997; Monteville *et al.*, 2003). These results may indicate that *C. coli* CadF is less functional than its *C. jejuni* counterpart.

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