Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization

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The binding of *Campylobacter jejuni* to fibronectin (Fn), a component of the extracellular matrix, is mediated by a 37 kDa outer-membrane protein termed CadF for *Campylobacter* adhesion to fibronectin. The specificity of *C. jejuni* binding to Fn, via CadF, was demonstrated using antibodies reactive against Fn and CadF. More specifically, the anti-CadF antibody reduced the binding of two *C. jejuni* clinical isolates to immobilized Fn by greater than 50%. Furthermore, a *C. jejuni* wild-type isolate, in contrast to the isogenic CadF mutant, was found to compete with another *C. jejuni* wild-type isolate for host cell receptors. Given the relationship between the pericellular Fn matrix and the cytoskeleton, the involvement of host cell cytoskeletal components in *C. jejuni* internalization was also examined. Cytochalasin D and mycalolide B microfilament depolymerizing agents resulted in a significant reduction in *C. jejuni* invasion. Studies targeting paxillin, a focal adhesion signalling molecule, identified an increased level of tyrosine phosphorylation upon *C. jejuni* infection of INT 407 cells. Collectively, these data suggest CadF promotes the binding of *C. jejuni* to Fn, which in turn stimulates a signal transduction pathway involving paxillin.

**INTRODUCTION**

The ability of pathogenic bacteria to bind to host tissues is important as it represents an early event in the establishment of an *in vivo* niche. In some instances, such binding is also a prerequisite for host cell invasion, where the organisms are protected from the humoral and cellular immune responses (Alrutz & Isberg, 1998; Isberg & Tran Van Nhieu, 1994; Roberts, 1990). An emerging theme among pathogenic micro-organisms is their ability to utilize host cell molecules during the infectious process to facilitate their binding and entry into host cells (Watarai *et al.*, 1996).

*Campylobacter jejuni* is a Gram-negative, microaerophilic bacterium, and is recognized as the most frequent cause of gastrointestinal disease in developed countries. The ability of *C. jejuni* to colonize the gastrointestinal tract of humans is proposed to be essential for disease (Allos & Blaser, 1995; Wassenaar & Blaser, 1999). Fauchère *et al.* (1986) found that *C. jejuni* isolated from individuals with fever and diarrhoea exhibited greater binding to epithelial cells than strains isolated from individuals without fever and diarrhoea. Numerous studies have been done to identify and characterize *C. jejuni* adhesins (De Melo & Pechère, 1990; Fauchère *et al.*, 1989; Jin *et al.*, 2001; Kelle *et al.*, 1998; Kervella *et al.*, 1993; Konkel *et al.*, 1997; McSweegan & Walker, 1986; Moser & Schröder, 1995; Moser *et al.*, 1992, 1997; Pei & Blaser, 1993; Pei *et al.*, 1998; Schröder & Moser, 1997). Based on these studies, *C. jejuni* appears to synthesize a number of adhesive molecules.

Fibronectin (Fn) is a 220 kDa glycoprotein that is present at regions of cell-to-cell contact in the gastrointestinal epithelium, thereby providing a potential binding site for pathogens (Quaroni *et al.*, 1978). Several bacterial pathogens, including *Staphylococcus aureus* (Kuusela, 1978; Rydén *et al.*, 1983), *Streptococcus pyogenes* (Jaffe *et al.*, 1996; Myhre & Kuusela, 1983), *Salmonella enteritidis* (Baloda *et al.*, 1985), *Escherichia coli* (Fröman *et al.*, 1984; Visai *et al.*, 1991), *Neisseria gonorrhoeae* (van Putten *et al.*, 1998), *Mycobacterium avium* (Schorey *et al.*, 1996) and *Treponema* species (Dawson & Ellen, 1990, 1994; Thomas *et al.*, 1985), bind Fn. We identified a 37 kDa outer-membrane protein in *C. jejuni* that mediates the binding of the organism to the extracellular matrix (ECM) component Fn (Konkel *et al.*, 1997). The cadF (*Campylobacter* adhesion to Fn) gene has thus far been found to be conserved among *C. jejuni* and *Campylobacter coli* isolates (Konkel *et al.*, 1999). *In vivo* studies have suggested that the CadF protein is required for the colonization of chickens by *C. jejuni* (Ziprin *et al.*, 1999).

Published work indicates a relationship between the pericellular Fn matrix and the cytoskeleton (Gumbiner, 1996; Miyamoto *et al.*, 1998; van der Flier & Sonnenberg, 2001). In mammalian cells, the actin cytoskeleton is...
necessary for a variety of cellular processes including control of cell-to-cell and cell-to-substrate interactions. Actin nucleation occurs at membrane-associated sites called focal adhesions. Focal adhesions are the sites at which the bundles of actin filaments (stress fibres) are cross-linked with membrane-associated adhesion molecules (e.g. integrins) and extracellular molecules (Gumbiner, 1996; Sarkar, 1999). The integrin molecules bind extracellularly to matrix components and intracellularly associate with protein complexes consisting of vinculin, talin, α-actinin, paxillin, tensin, zyxin and focal adhesion kinase (FAK) (Miyamoto et al., 1998; Tachibana et al., 1995). The integrins are transmembrane glycoprotein receptors composed of heterodimeric αβ subunits (Danen & Yamada, 2001; Hynes, 1992; van der Flier & Sonnenberg, 2001; Vuori, 1998). The two subunits are noncovalently associated with one another (1:1) in the membrane. There are 18 known α subunits and 8 known β subunits (van der Flier & Sonnenberg, 2001). Different α and β subunit combinations dictate the specificity of cell-to-cell and cell-to-ECM recognition. The αβ3 integrin receptor specifically binds Fn (Hynes, 1992; Miyamoto et al., 1998). Integrin occupancy and clustering is associated with tyrosine phosphorylation of cellular cytoplasmic proteins including FAK and paxillin, and is a means of regulating host signal transduction events leading to actin rearrangements (Miyamoto et al., 1998; Tachibana et al., 1995). As stated earlier, certain bacterial pathogens are known to utilize host cell ECM and cytoskeleton components to their benefit. For example, N. gonorrhoeae appears to utilize a Fn-mediated uptake pathway involving integrin receptors (van Putten et al., 1998). The binding of N. gonorrhoeae to Fn is proposed to trigger the uptake of the organism via integrin receptors by stimulating the host cell signalling pathways that are responsible for cytoskeletal rearrangement.

Because C. jejuni isolates possess a minimum of three different adhesive molecules including CadF, JlpA and PEB1 (Jin et al., 2001; Konkel et al., 1997; Pei et al., 1998), we sought to determine the step of the infectious process in which the CadF outer-membrane protein participates. We investigated the binding properties of two C. jejuni clinical isolates, F38011 and 81-176, to the INT 407 cell line (human embryonic intestinal cells) and the participation of the INT 407 cells in C. jejuni uptake. While the host cell cytoskeletal components involved in C. jejuni F38011 uptake have not been examined previously, C. jejuni 81-176 has been reported to be internalized via a novel pathway exclusively involving microtubules (Bacon et al., 2000; Hu & Kopecko, 1999, 2000; Kopecko et al., 2001; Oelschlaeger et al., 1993). Based on the experiments performed herein implicating microfilaments in C. jejuni uptake, additional experiments were done to determine if the host cell signalling events known to be associated with cytoskeletal rearrangement occur during C. jejuni entry.

METHODS

Bacterial isolates and growth conditions. C. jejuni F38011, 81116 and 81-176 (TetR) wild-type clinical isolates were cultured on Mueller–Hinton agar plates containing 5 % bovine citrated blood (MH/blood) at 37 °C under microaerophilic conditions. C. jejuni 81-176 (TetR) was cultured on plates supplemented with 12.5 μg tetracycline ml⁻¹ (Bacon et al., 2000). C. jejuni F38011 isogenic cadF and Cj1477c mutants were cultured on MH/blood agar plates supplemented with 200 μg kanamycin ml⁻¹. The C. jejuni F38011 (StrepR/NalR) isolate was cultured on MH/blood agar plates supplemented with 200 μg streptomycin ml⁻¹ and 50 μg nalidixic acid ml⁻¹. Isolates were passaged every 24–48 h. Citrobacter freundii 8090 and Salmonella enterica subsp. Typhimurium (S. typhimurium) SL1344 were cultured aerobically on Luria–Bertani (LB) agar plates at 37 °C.

Binding of C. jejuni to immobilized ECM. Binding of C. jejuni isolates to human plasma Fn was assessed as previously described (Konkel et al., 1997). Specificity of binding was determined by preincubating Fn-coated coverslips with a 1:50 dilution of a rabbit anti-human Fn antibody (Telios Pharmaceuticals), preincubating C. jejuni isolates with a 1:50 dilution of a goat anti-C. jejuni 37 kDa serum, or by the addition of 100 μg Fn ml⁻¹. For each coverslip, the bacteria in each of three randomly chosen fields were counted.

Gel electrophoresis and immunoblot analysis. Bacterial whole-cell extracts (an equivalent of 0–1 OD₆₀₀ units) were solubilized in single strength electrophoresis sample buffer and incubated at 95 °C for 5 min. Proteins were separated in SDS-12.5 % PAGE minigels as previously described (Laemmli, 1970) and electrophoretically transferred to PVDF membranes (Immobilon P; Millipore). The membranes were washed three times in PBS and incubated for 18 h at 4 °C with a 1:500 dilution of a goat anti-C. jejuni 37 kDa serum in PBS pH 7.4/0.01 % Tween-20 containing 20 % foetal bovine serum (FBS). Bound antibodies were detected using peroxidase-conjugated rabbit anti-goat IgG (Sigma) at a 1:2000 dilution and 4-chloro-1-naphthol (Sigma) as the chromogenic substrate.

Bacterial concentration of gentamicin. Assays were performed to determine the concentration of gentamicin and length of incubation time required to kill each isolate used in this study. C. jejuni isolates, C. freundii and S. typhimurium were suspended in Minimal Essential Medium (MEM) (Gibco Invitrogen) supplemented with 1 % FBS (MEM-1 % FBS) or 10 % FBS (MEM-10 % FBS) at an approximate concentration of 2×10⁶ c.f.u. ml⁻¹. Aliquots (1 ml) of the bacterial suspensions were placed in a 24-well plate and incubated at 37 °C in a humidified, 5 % CO₂ incubator for 2 h. Gentamicin (Life Technologies) was added to the media at a final concentration of 0, 100, 250, 500 and 750 μg ml⁻¹, and the suspensions again incubated as above. Following incubation (2 or 3 h), the bacterial suspensions (1 ml) were removed and bacteria pelleted by centrifugation at 6000 g for 10 min. The supernatant fluids were discarded to remove the gentamicin and bacteria resuspended in 1 ml PBS. Suspensions were serially diluted and the number of viable bacteria assessed. Each isolate was tested at least three times in triplicate.

INT 407 binding and internalization assays. A stock culture of INT 407 cells (human embryonic intestine, ATCC CCL 6) was obtained from the American Type Culture Collection. This cell line was cultured in MEM-10 % FBS at 37 °C in a humidified, 5 % CO₂ incubator. For experimental assays, each well of a 24-well tissue culture tray was seeded with 1×10⁶ cells per well and incubated for 18 h at 37 °C in a humidified, 5 % CO₂ incubator. The cells were rinsed with MEM-1 % FBS and inoculated with approximately
5 × 10² c.f.u. of a bacterial suspension. Tissue culture trays were centrifuged at 600 g for 5 min, and incubated at 37 °C in a humidified, 5 % CO₂ incubator. For binding, the infected monolayers were incubated for 2 h, rinsed three times with PBS and the epithelial cells lysed with a solution of 0.1 % (v/v) Triton X-100 (Calbiochem). The suspensions were serially diluted and the number of viable, adherent bacteria determined by counting the resultant colonies on MH/blood plates. To measure bacterial internalization, the infected monolayers were incubated for 2 h, rinsed three times with MEM-1% FBS, and incubated for an additional 3 h in MEM-1% FBS containing a bactericidal concentration of gentamicin. The number of internalized bacteria was determined as outlined above. Unless otherwise stated, the reported values represent the mean counts ± SD derived from triplicate wells. All assays in this study using cytoskeletal inhibitors were performed at a m.o.i. ranging between 50 and 500 to ensure reproducibility, and repeated a minimum of three times. Regardless of whether a m.o.i. of 50 to 1 or 500 to 1 was used, the effect of the cytoskeletal inhibitory drugs was the same.

**Competitive inhibition assays.** INT 407 cells were inoculated with a suspension containing the C. jejuni 81-176 (Tet<sup>+</sup>) isolate and the C. jejuni cadF mutant (Kan<sup>R</sup>) as well as the C. jejuni 81-176 (Tet<sup>+</sup>) isolate and the C. jejuni F38011 (Strep<sup>R</sup>/Nal<sup>R</sup>) isolate. Binding assays were performed as mentioned above except that a m.o.i. of approximately 10 was used for the C. jejuni 81-176 (Tet<sup>+</sup>) wild-type isolate. To determine the number of bacteria of a particular isolate that became bound, serial dilutions of the suspensions were plated on MH/blood agar plates supplemented with the appropriate selective antibiotic. No evidence of horizontal gene transfer was apparent as judged by lack of recovery of Strep<sup>R</sup>/Nal<sup>R</sup>/Tet<sup>R</sup> isolates upon mixing experiments with the C. jejuni 81-176 (Tet<sup>+</sup>) and C. jejuni F38011 (Strep<sup>R</sup>/Nal<sup>R</sup>/Tet<sup>R</sup>) isolates.

**Inhibitor studies.** INT 407 cells were preincubated for 45 min in MEM-1% FBS with nocodazole, cytochalasin D or mycocalide B at the concentrations indicated in the text. When performing experiments looking at the combined effects of inhibitors, nocodazole and cytochalasin D were both added to INT 407 cells during the preincubation step. Following incubation, cells were infected with approximately 5 × 10² c.f.u. of each isolate while maintaining indicated inhibitor concentrations. S. typhimurium was preincubated anaerobically at 37 °C for 3 h prior to infection. Binding and internalization assays were performed as outlined above. The values reported represent the percentage of adherent and internalized bacteria relative to the control (untreated sample) or the mean ± SD of adherent and internalized bacteria. INT 407 cell viability, following inhibitor treatment, was assessed by rinsing the INT 407 cells twice with PBS and staining the cells for 5 min with 0.5% trypsin blue. The cells were then rinsed twice with PBS and counter-stained for 5 min with 0.5 ml 0.5% phenol red (Humason, 1979). The INT 407 cells were visualized with an inverted microscope.

**Reversibility of cytochalasin D.** INT 407 cells were pretreated with cytochalasin D and infected with bacteria as described above. To assess the reversibility of cytochalasin D, the INT 407 cell monolayers were rinsed after 2 h with MEM-1% FBS. A set of the cytochalasin D-treated cells were then incubated for an additional 2 h in MEM-1% FBS containing cytochalasin D or in the presence of medium alone. The number of adherent and internalized bacteria was determined as outlined above.

**Preparation of the polyclonal antisera.** Female New Zealand White rabbits were subcutaneously injected with 100 μg of each bacterial whole-cell extract in TiterMax Gold (CytRx Corporation). A booster injection of 50 μg whole-cell extract in Freund’s incomplete adjuvant (Sigma) was given after 4 weeks. Blood was collected prior to first and second immunizations, and 2 weeks after the second immunization. Sera were stored at −20 °C.

**Confocal microscopy examination of C. jejuni infected cells.** INT 407 cells (5 × 10⁴ cells) were cultured on 13 mm circular glass coverslips for 18 h at 37 °C in a humidified, 5 % CO₂ incubator. The cells were infected by the addition of 0–5 ml of a bacterial suspension (1 × 10⁷ c.f.u. per well) in MEM. Mock-infected cells were used in certain instances as a negative control. Prior to infection, cell monolayers were rinsed once with MEM. Following incubation (45 min), the cell monolayers were rinsed three times with PBS and fixed with 3 % glutaraldehyde or 3 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Cells were permeabilized with 0.1 % (v/v) Triton-X 100. Tubulin was stained using a 1:250 dilution of FITC-conjugated monoclonal antibody against tubulin (clone DM 1A; Sigma). Actin was stained using FITC-labelled phalloidin (Sigma) at a concentration of 0.4 μg ml⁻¹. Primary antibodies directed towards bacteria were used at a 1:250 dilution followed by a secondary goat anti-rabbit IgG-rhodamine F(AB’)<sub>2</sub> fragment antibody at a 1:500 dilution.

**Immunoprecipitation.** INT 407 cells were cultured in six-well tissue culture trays as outlined above. INT 407 cell monolayers were rinsed once with MEM and inoculated with a suspension of the C. jejuni F38011 wild-type isolate and the isogenic cadF mutant (m.o.i., 100). Tissue culture trays were centrifuged and incubated as stated previously. At timepoints indicated, cell monolayers were rinsed three times with PBS and lysed by the addition of ice-cold lysis buffer (125 mM Tris/HCl pH 8.0, 137 mM NaCl, 10 % glycerol, 0.5–5 % sodium deoxycholate, 1 % NP-40, 2 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄ and 1:76 Trypsin Inhibitor Units aprotinin). The insoluble and soluble fractions were separated by centrifugation at 14,000 g for 10 min at 4 °C. Immunoprecipitation was performed by adding a mouse anti-paxillin antibody (Clone 349, Transduction Labs) to the soluble fraction and incubating at 4 °C for 2 h with end-over-end mixing. Following mixing, prewashed protein G agarose beads (Gibco-BRL) were added and samples mixed for an additional 2 h at 4 °C. The precipitate was rinsed four times in ice-cold lysis buffer and once with PBS. Samples were analysed by SDS-PAGE and immunoblot analysis as outlined above. Immunoblot detection of phosphorylated paxillin was performed using a 1:2000 dilution of a mouse anti-phosphotyrosine antibody (PT-66; Sigma) and a 1:80 000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG by enhanced chemiluminescence (Renaissance; NEN Life Science Products). Detection of the total pool of paxillin was performed using a 1:10 000 dilution of a mouse anti-paxillin antibody and a 1:80 000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG.

**Other analytical procedures.** To determine the MIC of gentamicin sulphate, C. jejuni strains 81-176, 81116 and F38011 were cultured on MH/blood agar plates for 24 h at 37 °C under microaerophilic conditions. Bacteria were suspended in 3 ml PBS (OD<sub>540</sub>=0–17), and 10 μl of each cell suspension was placed onto a MH/blood agar plate containing gentamicin sulphate. Gentamicin concentrations ranged from 1 μg ml⁻¹ to 512 μg ml⁻¹. Before and after spotting the suspensions on the antibiotic containing plates, 10 μl of each cell suspension was also placed on a MH/blood agar plate without antibiotic to ensure that bacterial viability was maintained. MICs were assessed by determining the lowest concentration of gentamicin in which no growth was observed following a 24 h incubation.

To ensure that the phenotype associated with the C. jejuni CadF mutant was due specifically to the mutation of the cadF gene, a knockout was generated in GJ1477c. GJ1477c lies downstream of the cadF gene in C. jejuni F38011, and encodes a putative hydrolase (GJ1477c).
RESULTS

CadF promotes the binding of C. jejuni to Fn

Previous work in our laboratory revealed that a 37 kDa outer-membrane protein, termed CadF, promoted the binding of C. jejuni F38011 to Fn (Konkel et al., 1997). To build on this initial work, experiments were performed to determine the role of CadF using the C. jejuni clinical isolates F38011 and 81-176. Consistent with our previous work showing that CadF is conserved among C. jejuni isolates (Konkel et al., 1997, 1999), immunoblot analysis with a goat anti-C. jejuni CadF serum revealed a 37 kDa reactive band in the whole cell lysates of both C. jejuni F38011 and 81-176 clinical isolates. A band corresponding to the 37 kDa CadF protein was not detected in the lysate of a C. jejuni F38011 cadF mutant (Fig. 1). The C. jejuni 81-176 wild-type isolate was also found to bind to Fn-coated coverslips (Table 1). The binding to Fn by both C. jejuni F38011 and 81-176 was judged to be specific because it was significantly reduced by an anti-Fn serum, an anti-CadF serum and the addition of exogenous Fn (Table 1). The C. jejuni cadF mutant bound to Fn at levels less than 10% of the C. jejuni F38011 and 81-176 clinical isolates. Additionally, no difference was noted in the binding of the C. jejuni cadF mutant to Fn-coated coverslips when compared to the BSA-coated coverslips. Collectively, these data suggest that CadF is the primary Fn-binding constituent in C. jejuni F38011 and 81-176.

Table 1. Competitive inhibition of C. jejuni binding to Fn with antibodies reactive against either Fn or CadF and upon the addition of exogenous Fn

<table>
<thead>
<tr>
<th>Substrate (40 μg)</th>
<th>Inhibitor</th>
<th>Bound bacteria (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. jejuni</td>
<td>F38011</td>
</tr>
<tr>
<td></td>
<td>C. jejuni</td>
<td>81-176</td>
</tr>
<tr>
<td>Fn</td>
<td>–</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>BSA</td>
<td>–</td>
<td>1-0 ± 0-3*</td>
</tr>
<tr>
<td>Fn</td>
<td>αFn</td>
<td>15 ± 3*</td>
</tr>
<tr>
<td>Fn</td>
<td>αCadF</td>
<td>41 ± 2*</td>
</tr>
<tr>
<td>Fn</td>
<td>Exogenous Fn</td>
<td>29 ± 16*</td>
</tr>
</tbody>
</table>

*The value was significantly different (P<0.01) from that obtained for the binding of the C. jejuni F38011 and 81-176 wild-type isolates to Fn in the absence of a potential inhibitor. Significance between samples was determined using Student’s t test following logarithmic (log10) transformation of the data.
**CadF is required for the maximal binding and internalization of *C. jejuni* to INT 407 cells**

To determine whether CadF plays a role in promoting bacteria–host cell interactions, binding and internalization assays were performed with INT 407 cells and the *C. jejuni* F38011 wild-type isolate and *cadF* mutant. At a m.o.i. of 30:1, the *C. jejuni* cadF knockout showed a 59% reduction in adherence to INT 407 cells when compared to the *C. jejuni* F38011 wild-type isolate (data not shown). To determine whether the phenotype displayed by the *C. jejuni* cadF knockout was solely due to the absence of CadF protein, a knockout was generated in Cj1477c. Cj1477c lies downstream of the *cadF* gene in *C. jejuni* F38011 and encodes a putative hydrolase (Cj1477c). In contrast to the *C. jejuni* cadF knockout was due solely to the absence of the CadF protein, and was not the result of indirect effects involving the expression of adjacent genes.

To determine if the *C. jejuni* 81-176 clinical isolate utilizes CadF as an adhesin for host cells, competitive inhibition assays were performed using the *C. jejuni* F38011 wild-type isolate and *C. jejuni* F38011 *cadF* mutant (Table 2). A significant decrease was noted in the number of *C. jejuni* 81-176 bound to the INT 407 cells in the presence of a 44- and 88-fold excess of the *C. jejuni* F38011 wild-type isolate. Moreover, increasing the number of competing organisms resulted in a dose-dependent decrease in the number of *C. jejuni* 81-176 bound. In contrast, a statistically significant difference was not observed in the number of *C. jejuni* 81-176 bound to the INT 407 cells in the presence of the *C. jejuni* cadF mutant. Based on these findings, we concluded that CadF serves as an adhesin for the *C. jejuni* F38011 and 81-176 clinical isolates.

**Table 2. The functional role of CadF as an adhesin is conserved in *C. jejuni* clinical isolate 81-176**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>No. <em>C. jejuni</em> 81-176 wild-type (TetR) isolate bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(3.0 ± 0.3)×10^5; 100%</td>
</tr>
<tr>
<td><em>C. jejuni</em> F38011</td>
<td>(2.5 ± 0.4)×10^5; 83 ± 14%*</td>
</tr>
<tr>
<td>44-fold excess</td>
<td>(2.3 ± 0.3)×10^5; 77 ± 10%</td>
</tr>
<tr>
<td>94-fold excess</td>
<td>(1.2 ± 0.2)×10^5; 40 ± 7%†</td>
</tr>
<tr>
<td>88-fold excess</td>
<td>(8.0 ± 1.4)×10^5; 27 ± 4%†</td>
</tr>
</tbody>
</table>

*Values represent the number of *C. jejuni* 81-176 bound in the presence of the competing organism divided by the number of *C. jejuni* 81-176 bound in the absence of the competitor, multiplied by 100.
†The value was significantly different (*P* < 0.01) from that obtained using the *C. jejuni* 81-176 wild-type isolate in the absence of the competitor. Significant differences were determined using Student’s *t* test following logarithmic (log_{10}) transformation of the data.

**C. jejuni** internalization involves actin cytoskeletal reorganization

Previous studies with *C. jejuni* 81-176 have suggested that this isolate is internalized via a unique pathway requiring only microtubules (Oelschlaeger et al., 1993). Because *C. jejuni* 81-176 binds to Fn, we re-examined the role of microfilaments in *C. jejuni* 81-176 binding and internalization in the presence of microfilament inhibitors. Cytochalasin D serves as an actin-capping compound that binds to the barbed end of actin filaments, thereby shifting the polymerization–depolymerization equilibrium leading to depolymerization of microfilaments (Cooper, 1987). *S. typhimurium* and *C. freundii* were used as controls (Biswas et al., 2000; Oelschlaeger et al., 1993). Prior to performing the assays, preliminary experiments were conducted to ensure that cytochalasin D, at the concentrations used here, had no effect on INT 407 cell viability. In addition, preliminary experiments were conducted with each bacterial isolate to ensure that a bactericidal concentration of gentamicin was used in the invasion assay. In contrast to previous reports (Bacon et al., 2000; Hu & Kopecko, 1999; Oelschlaeger et al., 1993), it was found necessary to treat *C. jejuni* 81-176 with a concentration of 500 μg gentamicin ml^-1^ for 3 h to kill the organism. A 2 h treatment of *C. jejuni* 81-176 (range 1.7–3.2×10^6 c.f.u.) with 100 μg gentamicin ml^-1^ resulted in the recovery of 4.8×10^6–2.9×10^5 c.f.u. (*n* = 4 independent experiments). The bactericidal activity of gentamicin was dependent on concentration and time of exposure while independent of the percentage of FBS in the medium (1% or 10%).

The effects of cytochalasin D on the *C. jejuni* F38011 and 81-176 clinical isolates, *S. typhimurium*, and *C. freundii* are shown in Table 3. Cytochalasin D resulted in a dose-dependent increase in binding and a dose-dependent decrease in internalization of both *C. jejuni* F38011 and 81-176 to INT 407 cells regardless of the m.o.i. used in an individual experiment. Consistent with previous reports (Bacon et al., 2000; Biswas et al., 2000; Hu & Kopecko, 1999, 2000; Oelschlaeger et al., 1993), cytochalasin D also significantly inhibited the internalization of *S. typhimurium* and *C. freundii* by INT 407 cells. Confocal microscopy examination of the infected INT 407 cells did not reveal convincing evidence for the interaction of *C. jejuni* with cellular microfilaments. However, microfilament supported structures were observed in contact with *C. jejuni* 81-176 (Fig. 2). Also noted was the expected interaction of *S. typhimurium* with a cellular structure supported by microfilaments.

To further investigate the role of microfilaments in *C. jejuni* internalization, assays were performed with mycalolide B. Mycalolide B severs microfilaments (F-actin), causing them...
to depolymerize, and sequesters G-actin, which inhibits microfilament polymerization (Saito et al., 1994, 1998; Wada et al., 1998). While mycalolide B was found to significantly inhibit the internalization of C. jejuni and S. typhimurium (Table 4), the drug had no effect on the viability of the INT 407 cells as judged by staining with trypan blue (data not shown). These data also suggest that the uptake of C. jejuni F38011, as well as 81-176, utilize microfilaments. Our finding of the requirement for actin rearrangement in S. typhimurium internalization is consistent with that of previous reports (Finlay et al., 1991; Francis et al., 1992, 1993).

### The effect of cytochalasin D on C. jejuni internalization is reversible

Because treatment of the INT 407 cells with cytochalasin D inhibited the uptake of each organism tested (C. jejuni F38011, C. jejuni 81-176, S. typhimurium and C. freundii), we questioned whether a small number of the INT 407 cells were being removed from the plastic substrate during the rinsing steps. If cells were detaching from the plastic substrate during either the drug-treatment step or the rinses, it could contribute to the decrease noted in internalized organisms. Thus, an assay was performed to examine the

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**Table 3. Effect of microfilament inhibitors on C. jejuni binding and internalization**

Assays were performed as outlined in Methods. Results are presented as the percentage of adherent and internalized bacteria relative to the values obtained for the wild-type isolates in the absence of the inhibitor.

<table>
<thead>
<tr>
<th></th>
<th>Adherent</th>
<th>Internalized</th>
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<tbody>
<tr>
<td><strong>C. jejuni F38011</strong></td>
<td>100; (5.5 ± 0.5)×10⁶</td>
<td>100; (6.0 ± 0.8)×10⁴</td>
</tr>
<tr>
<td>Adherent</td>
<td>96 ± 11</td>
<td>82 ± 3*</td>
</tr>
<tr>
<td>Internalized</td>
<td>149 ± 7*</td>
<td>60 ± 5*</td>
</tr>
<tr>
<td><strong>C. jejuni 81-176</strong></td>
<td>100; (9.8 ± 0.9)×10⁶</td>
<td>100; (4.2 ± 0.3)×10⁵</td>
</tr>
<tr>
<td>Adherent</td>
<td>128 ± 10*</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>Internalized</td>
<td>151 ± 11*</td>
<td>65 ± 3*</td>
</tr>
<tr>
<td><strong>S. typhimurium SL1344</strong></td>
<td>100; (1.3 ± 0.2)×10⁶</td>
<td>100; (8.5 ± 0.6)×10⁴</td>
</tr>
<tr>
<td>Adherent</td>
<td>89 ± 11</td>
<td>15 ± 3*</td>
</tr>
<tr>
<td>Internalized</td>
<td>112 ± 23</td>
<td>2.8 ± 0.4*</td>
</tr>
<tr>
<td><strong>C. freundii 8090</strong></td>
<td>100; (6.8 ± 0.9)×10⁶</td>
<td>100; (1.3 ± 0.2)×10⁴</td>
</tr>
<tr>
<td>Adherent</td>
<td>88 ± 11</td>
<td>4.3 ± 0.5*</td>
</tr>
<tr>
<td>Internalized</td>
<td>94 ± 11</td>
<td>1.8 ± 0.4*</td>
</tr>
</tbody>
</table>

*The value was significantly different (P<0.01) from that obtained using the wild-type isolate in the absence of the inhibitor. Significance between samples was determined using Student’s t test following logarithmic (log_10) transformation of the data.

**Table 4. Effect of mycalolide B microfilament inhibitor on C. jejuni binding and internalization**

Assays were performed as outlined in Methods. Results are presented as the percentage of adherent and internalized bacteria relative to the values obtained for the wild-type isolates in the absence of the inhibitor. ND, Not done.

<table>
<thead>
<tr>
<th></th>
<th>Adherent</th>
<th>Internalized</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. jejuni F38011</strong></td>
<td>100; (1.1 ± 0.1)×10⁷</td>
<td>100; (1.2 ± 0.1)×10⁵</td>
</tr>
<tr>
<td>Adherent</td>
<td>102 ± 19</td>
<td>85 ± 9</td>
</tr>
<tr>
<td>Internalized</td>
<td>121 ± 8</td>
<td>69 ± 8*</td>
</tr>
<tr>
<td><strong>C. jejuni 81-176</strong></td>
<td>100; (7.5 ± 1.3)×10⁶</td>
<td>100; (6.6 ± 0.4)×10⁵</td>
</tr>
<tr>
<td>Adherent</td>
<td>103 ± 13</td>
<td>82 ± 9</td>
</tr>
<tr>
<td>Internalized</td>
<td>110 ± 13</td>
<td>60 ± 3*</td>
</tr>
<tr>
<td><strong>S. typhimurium SL1344</strong></td>
<td>100; (7.5 ± 0.5)×10⁵</td>
<td>100; (6.4 ± 0.5)×10⁵</td>
</tr>
<tr>
<td>Adherent</td>
<td>68 ± 9*</td>
<td>41 ± 8*</td>
</tr>
<tr>
<td>Internalized</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The value was significantly different (P<0.01) from that obtained using the wild-type isolate in the absence of the inhibitor. Significance between samples was determined using Student’s t test following logarithmic (log_10) transformation of the data.
reversibility of the effect caused by cytochalasin D. The effect of cytochalasin D on C. jejuni internalization was reversible, indicating that the INT 407 cells were not detaching from the plastic substrate (Table 5). In addition, the increase in adherent bacteria caused by the drug treatment resulted in an increase in internalized organisms following the drug’s removal from the assay medium, indicating that the adherent bacteria are capable of becoming internalized.

**C. jejuni internalization is sensitive to microtubule inhibitors**

Binding and internalization assays were also performed with the C. jejuni F38011 and 81-176 clinical isolates, S. typhimurium and C. freundii in the presence of the microtubule inhibitor nocodazole (Table 6). Nocodazole binds β-tubulin, preventing tubulin polymerization, as well as enhances GTPase activity (Mejillano et al., 1996). Nocodazole had no effect on the binding of the C. jejuni isolates to the INT 407 cells, but did significantly inhibit the uptake of both isolates by the INT 407 cells in a dose-dependent fashion. While the internalization of C. freundii was also significantly inhibited by nocodazole, the drug had no effect on S. typhimurium internalization. A clear association of C. jejuni with microtubules was not observed by confocal microscopy examination of the infected INT 407 cells (Fig. 3). Others have suggested that C. jejuni can be internalized via two distinct uptake pathways where microtubules and microfilaments act exclusively (Hu & Kopecko, 1999; Kopecko et al., 2001; Oelschlaeger et al., 1993). Because the uptake of the C. jejuni isolates tested was found to be sensitive to both microfilament and microtubule depolymerizing agents, assays were performed to determine the combined effects of these drugs on C. jejuni internalization (Table 7). When the drugs were used in combination, the number of C. jejuni bound to the INT 407 cells was comparable to that obtained when only cytochalasin D was used. Significant differences were not observed in the number of C. jejuni internalized by the INT 407 cells in the presence of either cytochalasin D or nocodazole alone versus when the drugs were used in combination with one another. As the combination of these two drugs on C. jejuni internalization was not additive, the data suggest that

<table>
<thead>
<tr>
<th>Table 5. Reversibility of cytochalasin D on C. jejuni binding and internalization</th>
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<tbody>
<tr>
<td>Assays were performed as outlined in Methods. Results are presented as the percentage of adherent and internalized bacteria relative to the values obtained for the wild-type isolates in the absence of the inhibitor.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1st incubation... No inhibitor</th>
<th>Cytochalasin D (2 μM)</th>
<th>Cytochalasin D (2 μM)</th>
</tr>
</thead>
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<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>C. jejuni F38011</strong></td>
<td>100; (3.1 ± 0.5)×10^6</td>
<td>168 ± 9*</td>
<td>168 ± 13*</td>
</tr>
<tr>
<td>Adherent</td>
<td>100; (3.6 ± 0.2)×10^4</td>
<td>50 ± 3*</td>
<td>128 ± 19</td>
</tr>
<tr>
<td>Internalized</td>
<td>100; (6.3 ± 0.5)×10^5</td>
<td>130 ± 16*</td>
<td>138 ± 10*</td>
</tr>
<tr>
<td><strong>C. jejuni 81-176</strong></td>
<td>100; (6.0 ± 0.8)×10^4</td>
<td>45 ± 8*</td>
<td>142 ± 8*</td>
</tr>
<tr>
<td>Adherent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internalized</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The value was significantly different (P<0.01) from that obtained using the wild-type isolate in the absence of the inhibitor. Significance between samples was determined using Student’s t test following logarithmic (log10) transformation of the data.

Fig. 2. Staining of microfilaments in INT 407 cells infected with C. jejuni, S. typhimurium and C. freundii. Bacteria were reacted with the appropriate polyclonal antibody and stained using a rhodamine-labelled secondary antibody. Microfilaments (e.g. F-actin) were stained using FITC-labelled phalloidin. (a) Uninfected INT 407 cells; (b) cytochalasin D (2 μM) treated INT 407 cells; (c) C. jejuni F38011; (d) C. jejuni 81-176; (e) S. typhimurium SL1344; (f) C. freundii 8090. The organism in (d) is indicated (arrow) to ease its visualization. Panels (a) and (b), and (c)–(f) were obtained under the same magnification.
microfilaments and microtubules act in concert to facilitate C. jejuni uptake (Table 7).

Maximal C. jejuni entry is accompanied by the tyrosine phosphorylation of paxillin

As the data indicate that C. jejuni uptake involves cytoskeletal rearrangement, it is likely that the uptake of the organism is accompanied by cytoskeletal signal transduction events. Therefore, experiments were performed to examine the levels of tyrosine phosphorylated paxillin in INT 407 cells immediately prior to and following inoculation with C. jejuni. An increased level of phosphorylated paxillin is an indicator of integrin stimulation (Watarai et al., 1996). Integrin molecules bind extracellularly to matrix components (e.g. Fn) and intracellularly associate with protein complexes that ultimately are linked to microfilaments. Here, the INT 407 cells were infected with the C. jejuni F38011 isolate at a m.o.i. of 100 to 1 and incubated for varying lengths of time, ranging between 15 and 60 min. Following incubation, the focal adhesion-associated protein paxillin was immunoprecipitated and subjected to SDS-PAGE coupled with immunoblot analysis using an anti-phosphotyrosine antibody. Infection of the INT 407 cells with the C. jejuni F38011 wild-type isolate led to an increase in the level of tyrosine phosphorylated paxillin at 30 and 45 min post-infection (Fig. 4). An increase in the level of phosphorylated paxillin was not observed in INT 407 cells infected with the C. jejuni cadF mutant until the m.o.i. was increased 20-fold beyond that of the wild-type isolate, after which the pattern of phosphorylated paxillin mirrored that observed for the wild-type isolate (data not shown). Consistent with paxillin phosphorylation, co-localization was observed between C. jejuni and host cell-tyrosine phosphorylated proteins by confocal microscopy (data not shown).

DISCUSSION

Binding of C. jejuni to epithelial cells is mediated by several outer-membrane proteins including JlpA, PEB1 and CadF (Jin et al., 2001; Konkel et al., 1997; Pei et al., 1998). Although it is difficult to assess the contribution of each adhesin in the adherence of C. jejuni to host cells given variations in assay protocols, knockouts have been generated in the genes encoding the JlpA, PEB1 and CadF adhesins, and the phenotypes examined. A C. jejuni jlpA knockout is reduced 19 % in adherence to HEp-2 cells (Jin et al., 2001). A C. jejuni peb1 knockout is reduced 10–99 %, depending on the initial inoculum, in adherence to HeLa cells (Pei et al., 1998). A C. jejuni cadF knockout is reduced 50–90 %, depending on the initial inoculum, in adherence to INT 407 cells. The biological significance of the CadF adhesin has also been demonstrated in vivo. Ziprin et al. (1999) reported that a C. jejuni F38011 cadF mutant is unable to colonize the intestinal tract of Leghorn chickens (n=60), thus providing evidence that the 37 kDa outer-membrane protein plays an in vivo role in mediating the organism’s binding to the intestinal epithelium. We undertook this study to more closely examine the role of CadF in C. jejuni–host cell interactions. Based on the results presented here, CadF appears to promote the binding of C. jejuni to Fn, thereby stimulating the host cell signalling events associated with bacterial uptake.

Two C. jejuni clinical isolates, F38011 (Lior serotype 90) and 81-176 (Lior serotype 5), were used in this study. The

| Table 6. Effect of microtubule inhibitors on C. jejuni binding and internalization |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Adherent        | Internalized    | Adherent        | Internalized    |
| C. jejuni F38011| 100; (5.5 ± 0.5)×10⁶| 96 ± 6          | 100 ± 11        | 96 ± 6          |
| C. jejuni 81-176| 100; (6.0 ± 0.8)×10⁶| 75 ± 7          | 52 ± 1*         | 40 ± 5*         |
| S. typhimurium SL1344| 100; (7.0 ± 1.0)×10⁶| 105 ± 13        | 84 ± 16         | 93 ± 14         |
| C. freundii 8090| 100; (6.5 ± 0.6)×10⁵| 88 ± 6          | 65 ± 8*         | 45 ± 9*         |
|                 | (Concn nocodazole (µM)) |               |                 |                 |
|                 | 0               | 1               | 10              | 20              |
| C. jejuni F38011| 100; (5.5 ± 0.5)×10⁶| 96 ± 6          | 100 ± 11        | 96 ± 6          |
| C. jejuni 81-176| 100; (6.0 ± 0.8)×10⁶| 75 ± 7          | 52 ± 1*         | 40 ± 5*         |
| S. typhimurium SL1344| 100; (7.0 ± 1.0)×10⁶| 105 ± 13        | 84 ± 16         | 93 ± 14         |
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|                 | (Concn nocodazole (µM)) |               |                 |                 |
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|                 | (Concn nocodazole (µM)) |               |                 |                 |
|                 | 0               | 1               | 10              | 20              |
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*The value was significantly different (P<0.01) from that obtained using the wild-type isolate in the absence of the inhibitor. Significance between samples was determined using Student’s t test following logarithmic (log₁₀) transformation of the data.
binding of \textit{C. jejuni} F38011 and 81-176 to Fn was inhibited by 54 and 56\%, respectively, with the anti-CadF antibody. Based on these findings, the CadF protein was concluded to mediate the binding of both \textit{C. jejuni} isolates to Fn. The adhesive nature of the CadF protein in \textit{C. jejuni} 81-176 was further revealed upon performing competitive inhibition binding assays with the \textit{C. jejuni} F38011 isolate and \textit{C. jejuni} F38011 cadF mutant. Here, only the \textit{C. jejuni} F38011 wild-type isolate was able to competitively inhibit the binding of \textit{C. jejuni} 81-176 to the INT 407 cells. Because Fn is associated with microfilaments, the role of microfilaments in \textit{C. jejuni} uptake was examined. Inhibitor studies revealed that the \textit{C. jejuni} F38011 and 81-176 isolates require microfilament participation for efficient host cell entry. The reduction of \textit{C. jejuni} uptake with cytochalasin D appeared specific as treatment of the INT 407 cells with this drug had no effect on INT 407 cell viability as judged by staining with trypan blue. Moreover, the effect of cytochalasin D, which inhibits actin polymerization and transient integrin-stimulated FAK activation (Schlaepfer \textit{et al.}, 1999), was reversible. Finally, treatment of the INT 407 cells with mycalolide B also inhibited \textit{C. jejuni} uptake. Mycalolide B severs microfilaments and sequesters G-actin. The sequestering of G-actin inhibits microfilament polymerization. Consistent with the notion that \textit{C. jejuni} uptake requires the stimulation of host cell signalling molecules, the amount of phosphorylated paxillin significantly increased 30 min after \textit{C. jejuni} infection. The amount of phosphorylated paxillin returned to a level equivalent to that of a non-infected control at the 1 h time point. The increase in phosphorylated paxillin slightly preceded and was concomitant with a sharp rise in the number of \textit{C. jejuni} internalized, which occurs 30–60 min post-infection (Konkel \textit{et al.}, 1993).

The inhibitory effect of microtubule-depolymerizing agents on the entry of \textit{C. jejuni} strain 81-176 has been noted previously (Bacon \textit{et al.}, 2000; Hu & Kopecko, 1999, 2000; Kopecko \textit{et al.}, 2001; Oelschlaeger \textit{et al.}, 1993). Based on this effect, the proposal has been put forth that \textit{C. jejuni} are internalized via two pathways, one involving microtubules.

The specificity of \textit{C. jejuni} binding to Fn, via CadF, was demonstrated using antibodies reactive against Fn and CadF, as well as by the addition of exogenous Fn. The binding of \textit{C. jejuni} F38011 and 81-176 to Fn was inhibited by 54 and 56\%, respectively, with the anti-CadF antibody. Based on these findings, the CadF protein was concluded to mediate the binding of both \textit{C. jejuni} isolates to Fn. The adhesive nature of the CadF protein in \textit{C. jejuni} 81-176 was further revealed upon performing competitive inhibition binding assays with the \textit{C. jejuni} F38011 isolate and \textit{C. jejuni} F38011 cadF mutant. Here, only the \textit{C. jejuni} F38011 wild-type isolate was able to competitively inhibit the binding of \textit{C. jejuni} 81-176 to the INT 407 cells. Because Fn is associated with microfilaments, the role of microfilaments in \textit{C. jejuni} uptake was examined. Inhibitor studies revealed that the \textit{C. jejuni} F38011 and 81-176 isolates require microfilament participation for efficient host cell entry. The reduction of \textit{C. jejuni} uptake with cytochalasin D appeared specific as treatment of the INT 407 cells with this drug had no effect on INT 407 cell viability as judged by staining with trypan blue. Moreover, the effect of cytochalasin D, which inhibits actin polymerization and transient integrin-stimulated FAK activation (Schlaepfer \textit{et al.}, 1999), was reversible. Finally, treatment of the INT 407 cells with mycalolide B also inhibited \textit{C. jejuni} uptake. Mycalolide B severs microfilaments and sequesters G-actin. The sequestering of G-actin inhibits microfilament polymerization. Consistent with the notion that \textit{C. jejuni} uptake requires the stimulation of host cell signalling molecules, the amount of phosphorylated paxillin significantly increased 30 min after \textit{C. jejuni} infection. The amount of phosphorylated paxillin returned to a level equivalent to that of a non-infected control at the 1 h time point. The increase in phosphorylated paxillin slightly preceded and was concomitant with a sharp rise in the number of \textit{C. jejuni} internalized, which occurs 30–60 min post-infection (Konkel \textit{et al.}, 1993).

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exclusively (considered a high efficiency uptake pathway) and the other involving microfilaments (considered a low efficiency uptake pathway) (Hu & Kopecko, 2000). While we noted a decrease in the invasiveness of C. jejuni 81-176 with a microtubule inhibitor, a decrease was also noted in the number of intracellular bacteria with this organism in the presence of microfilament inhibitors. The discrepancy between our results and those reported earlier is most likely due to differences in assay protocols. More specifically, in previous work, a 2 h incubation with 100 μg gentamicin ml\(^{-1}\) was used to kill the extracellular bacteria. In our hands, treatment of C. jejuni 81-176 with 100 μg gentamicin ml\(^{-1}\) for 2 h typically resulted in the recovery of 3–12 % of the bacteria in the original suspension (n=4 individual experiments). Thus, even though C. jejuni 81-176 is gentamicin-sensitive (e.g. MIC of 4 μg ml\(^{-1}\)), it was found necessary to increase both the time of exposure and the concentration of the antibiotic to ensure bacterial death. The protocol used by others to determine the bactericidal concentration of gentamicin was not reported (Bacon et al., 2000; Hu & Kopecko, 1999, 2000; Oelschlaeger et al., 1993).

Also noteworthy is that an increase has been noted in the number of C. jejuni 81-176 internalized in the presence of cytochalasin D (Hu & Kopecko, 1999, 2000; Oelschlaeger et al., 1993). One reason for the increase in C. jejuni 81-176 uptake noted by others with cytochalasin D may be due to an increase in the number of organisms bound to the host cells. Increasing the number of cell-associated bacteria while not using a bactericidal concentration of antibiotic would mask any effect of cytochalasin D on C. jejuni uptake. While this explanation seems possible, we do not know the number of cell-associated (adherent) bacteria in earlier studies in which the effect of cytochalasin D on C. jejuni 81-176 uptake was examined as it was not reported. Regardless, a sublethal concentration of gentamicin would not alter the net result of an internalization assay unless there was a significant increase or decrease in the number of adherent bacteria in the test sample versus the control.

To the best of our knowledge, C. jejuni 81-176 is the only isolate to date that has been reported to be internalized exclusively via a microtubule-dependent pathway (Kopecko et al., 2001; Oelschlaeger et al., 1993). Using 250 μg gentamicin ml\(^{-1}\) for 3 h to kill the extracellular bacteria, Biswas et al. (2000) observed a reduction in entry with both microfilament and microtubule inhibitors with every C. jejuni clinical isolate tested (n=9). Biswas et al. (2000) concluded that the most invasive isolates examined in their study utilized microfilaments. Based on the data currently available, the most reasonable conclusion is that C. jejuni uptake involves cooperation of both microfilaments and microtubules. More specifically, uptake of C. jejuni 81-176 and F38011 by INT 407 cells was reduced from 56 to 66 %, respectively, in the presence of nocodazole and cytochalasin D relative to the untreated control (Table 7). These results are consistent with those reported elsewhere, even though the experimental protocols vary (Biswas et al., 2000; Oelschlaeger et al., 1993). What is unclear is why in the presence of both inhibitors a significant number of C. jejuni are still internalized. In comparison, Salmonella invasion was reduced by greater than 99 % by cytochalasin D compared to untreated INT 407 cells. Comparable results for Salmonella have been reported by others (Bacon et al., 2000; Biswas et al., 2000; Hu & Kopecko, 1999, 2000; Oelschlaeger et al., 1993).

The mechanism by which treatment of cells with microtubule inhibitors causes a reduction in C. jejuni-cell uptake is not known. However, it has been reported that microtubules regulate the turnover of focal adhesion contacts and modulate a cell’s adhesive strength to the ECM (Ballestrem et al., 2000). In fact, treatment of cells with microtubule inhibitors leads to an increase in a cell’s adherence to the ECM (Ballestrem et al., 2000; Sastry & Burridge, 2000). Thus, the effect of a microtubule inhibitor on C. jejuni-cell uptake could be indirect, as the turnover of the focal adhesion sites is retarded. Alternatively, following initial microfilament-dependent uptake at the level of the plasma membrane, microtubules may be required for subsequent trafficking of the endosome to the interior of the cell as has been proposed for C. freundii (Badger et al.,
Regardless, there is clearly functional cooperation between host cytoskeletal elements (Ballestrem et al., 2000; Goode et al., 2000; Sastry & Burridge, 2000). Because the effects of cytochalasin D and nocodazole on *C. jejuni* uptake were not additive when used together, it appears most likely that the *C. jejuni* isolates tested here utilize microfilaments and microtubules together.

*C. jejuni* strain 81-176 has recently been reported to harbour two plasmids, one of which harbours the tetracycline resistance gene (*tetO*) and the other harbouring a gene termed *virB11* (Bacon et al., 2000). Moreover, in a recent review by Kopecko et al. (2001), the authors suggested a possible correlation between the plasmid-borne genes and the microtubule-dependent uptake pathway. To ensure that the strain of *C. jejuni* 81-176 used in this study harboured both plasmids, the isolate was subjected to PCR using *tetO* and *virB11* gene-specific primers. The identity of the amplified products was subsequently confirmed upon sequencing of the PCR-amplified products (not shown).

Wooldridge et al. (1996) previously reported that *C. jejuni* uptake is reduced in the presence of protein tyrosine phosphorylation inhibitors. We chose to examine whether paxillin was phosphorylated upon infection of INT 407 cells with *C. jejuni* because protein tyrosine phosphorylation is one of the earliest events upon integrin stimulation (Clark & Brugge, 1995). Consistent with the idea that the binding of *C. jejuni* leads to integrin stimulation, an increase in phosphorylated paxillin was observed 30–45 min after *C. jejuni* F38011 infection. Noteworthy is that the increase in phosphorylated paxillin occurs just prior to and concomitant with an increase in *C. jejuni* internalization (Konkel et al., 1993). In contrast to the *C. jejuni* wild-type isolate, an increase was not observed in phosphorylated paxillin over the course of the assay with cells inoculated with the *C. jejuni cadF* mutant at a m.o.i. of 100 to 1 (Fig. 4). However, upon infection of the INT 407 cells with the *C. jejuni cadF* mutant at a m.o.i. of greater than 2000 to 1, the pattern of phosphorylated paxillin in cells inoculated with the *C. jejuni cadF* mutant mirrored that obtained with the *C. jejuni* wild-type isolate. A possible explanation for this finding is that *C. jejuni* adherence is multifactorial, and that several adhesins simultaneously function to promote host cell binding, after which cell signalling events are stimulated. Thus, the CadF protein does not appear to be required to induce host cell signalling events, but appears to promote signalling events by facilitating the organism’s binding to appropriate host cells receptors. Noteworthy is that Watarai et al. (1996) observed an increase in the tyrosine phosphorylation of FAK and paxillin 20–30 min after infection of Chinese hamster ovary cells with *Shigella flexneri*.

In summary, the data suggest that CadF promotes *C. jejuni*–host cell interactions. Consistent with the notion that bacterial uptake requires host cell signalling, an increase in tyrosine phosphorylated paxillin was observed upon infection of INT 407 cells with *C. jejuni*. Tyrosine phosphorylation of FAK and paxillin is a means of regulating host signal transduction events leading to actin rearrangement (Miyamoto et al., 1998; Tachibana et al., 1995). Because paxillin is an integral component of focal adhesions, as are ECM components including Fn, we speculate that CadF is responsible for promoting the initial interaction of *C. jejuni* with the appropriate host cell receptors involved in uptake. Future studies will be directed toward the identification and biochemical characterization of the CadF Fn-binding domain(s).

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