Enhanced Microscopic Definition of *Campylobacter jejuni* 81-176 Adherence to, Invasion of, Translocation across, and Exocytosis from Polarized Human Intestinal Caco-2 Cells

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*Campylobacter jejuni* is a leading bacterial cause of human diarrheal disease. The U.S. Centers for Disease Control and Prevention estimates that *C. jejuni* causes illness annually in 1% of the population in the United States. *Campylobacter* infection usually causes watery to bloody diarrhea with fever, abdominal cramps, and the presence of fecal leukocytes. The results of intestinal biopsies of patients, infected primates, and several other experimental model animals, as well as cell culture assays, have demonstrated that *C. jejuni* is able to invade enterocytes and suggest that gut adherence, invasion, and mucosal translocation are important steps in pathogenesis (20, 29, 46).

The intestinal mucosa forms a barrier that protects against invasion of the host by nonpathogenic bacteria residing in the intestinal lumen. Some enteropathogens, such as *Salmonella, Shigella*, and *Yersinia*, have specific tissue-invading capabilities and can physically breach the intestinal mucosal barrier (32). Pathogen-induced traversal of the human intestinal mucosa may involve crossing of absorptive enterocytes or passage through the specialized M cells (14–16). In addition to transcellular entry, some bacteria can disrupt the tight junctions between enterocytes and cross via paracellular mechanisms (47). *Salmonella enterica* serovar Typhimurium may also cross the intestinal mucosa by sequestering itself within luminal CD18-positive mononuclear cells that shuttle the microbe paracellularly across the villus epithelium into the systemic circulation (48). *Shigella* (45) and possibly *Campylobacter* (49) may also breach the intestinal barrier by transcytosis across M cells. *C. jejuni* has previously been reported to undergo transcellular translocation across the mucosa; there have also been reports of paracellular translocation of *Campylobacter* (7, 9–12, 25, 28, 35, 47, 49).

Cultured mammalian cells are commonly used as a simple model for investigating the host epithelial cell-bacterium interaction that is more easily controlled than whole animals. Caco-2 cells were derived from transformed human colonic carcinoma cells. During 7 to 10 days in culture, these cells form polarized monolayers that have intercellular tight junctions and defined apical and basolateral surfaces. The differentiated apical surface has dense brush border microvilli containing normal intestinal brush border enzymes and antigens (13, 18, 43, 44). A key advantage of differentiated Caco-2 cells is that they provide a substrate monolayer similar to that for which *Campylobacter* shows a natural tropism in vivo. This cell line allows assessment of the ability of *C. jejuni* to penetrate and pass through a differentiated intestinal epithelial barrier, a process that appears to be important in causing overall colonic damage and the...
occasional bacteremia associated with Campylobacter enterocolitis (9, 10, 26, 28).

C. jejuni 81-76 is a well-characterized strain which exhibits relatively high levels of epithelial cell invasion in cultured host cell assays and causes a bloody colitis, as demonstrated in human challenge studies (6; D. Trible, unpublished data). Cell culture invasion assays conducted with inhibitors of cytoskeletal function initially demonstrated that this strain invades epithelial cells apically via a novel mechanism that is strongly microtubule (MT) dependent (38). Subsequent immunofluorescence studies of this 81-176 invasion process confirmed the involvement of MTs, demonstrated that the MT-associated molecular motor dynein is required for this uptake process, and defined the kinetics of INT407 cell invasion by C. jejuni 81-176 (19). Recent work has also shown that Ca2+ release from host intracellular stores is essential for C. jejuni 81-176 internalization into host cells (21). In addition, host signal transduction studies have suggested that strain 81-176 interacts at filipin III-sensitive membrane microdomains of the host cell surface with G-protein-coupled receptors, a process which activates phosphatidylinositol 3-kinase and mitogen-activated protein kinases that appear to be intimately involved in events controlling 81-176 internalization (22). Very recently, the rho GTPases Rac1 and Cdc42 have been implicated in the 81-176 invasion process (31). It is also noteworthy that other researchers, using different invasion assay conditions, have observed mixed involvement of both MTs and microfilaments in 81-176 invasion (5, 36). Watson and Galan (51) recently demonstrated that following internalization into host cells C. jejuni 81-176 survives within modified endosomes that avoid fusion with lysosomes.

Based on previous Campylobacter pathogenesis studies with cultured cells, animals, and humans (4–8, 10, 12, 17, 19–22, 23–40, 46, 49–53), we hypothesize that during the first few hours of infection C. jejuni adheres to and crosses differentially internated intestinal epithelial cells by a transcellular process which does not disrupt transepithelial electrical resistance (TER) (transcytosis is apical endocytosis followed by basolateral exocytosis). Thus, a kinetic examination of C. jejuni internalization and microscopic analyses of the events involving adherence, invasion, and transcytosis over a 4-h period were conducted with the goal of obtaining an enhanced understanding of Campylobacter-host cell interactions. Importantly, this study provided new information about entry kinetics and revealed new specific bacterium-host cell interactions involved in epithelial cell translocation that have not been observed in previous electron microscope (EM) studies of Campylobacter entry mechanisms (4, 10, 26, 28, 31).

MATERIALS AND METHODS

Bacterial strains, cell lines, media, and culture conditions. The experiments were conducted using C. jejuni 81-176, a well-studied strain that was originally obtained from a Campylobacter disease outbreak in Minnesota (30). A Δ cheY mutant of 81-176 designated RY213 (53), which is basically nonadherent and noninvasive, was used as a nonadherent control. Also, genome-sequenced strain NCTC 11168 was employed for adherence comparisons. C. jejuni was grown in Mueller-Hinton biphase medium and on Mueller-Hinton agar (Difco) under a Campylobactor microaerophilic atmosphere containing 10% O2, 5% CO2, and 85% N2. Immortalized human colon cancer cells (Caco-2) and human embryonic intestinal epithelial cells (INT407), obtained from the American Type Culture Collection, were maintained in liquid nitrogen and cultured in minimal essential media with 20% (for Caco-2 cells) or 10% (for INT407 cells) heat-inactivated fetal calf serum (FCS) (Gibco), 2 mM L-glutamine, and 0.1 mM nonessential amino acids, as recommended by the American Type Culture Collection. The relative state of cell differentiation and maintenance of tight junctions were monitored by determining monolayer TER with a dual-voltage ohmmeter (Millicell-ERS; Millipore) (3), and brush border organization was verified by transmission electron microscopy (EM) studies of C. jejuni 81-176 and NCTC 11168 was employed for adherence comparisons.

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RESULTS

C. jejuni invasion efficiencies with undifferentiated and differentiated host cells. Caco-2 cells cultured in 24-well plates for 1, 7, or 15 days were infected with C. jejuni 81-176 at different MOIs, and the invasion was compared to invasion of young INT407 cells. Caco-2 cells differentiated significantly and formed tight junctions by 7 days in culture, as indicated by the development of TER typically greater than 1,000 Ω/cm². Host cells were incubated initially for 2 h in the presence of C. jejuni, which was followed by washing and then incubation for another 2 h in the presence of gentamicin prior to enumeration of internalized bacteria. The highest efficiency of invasion of Caco-2 cells (2%) was observed at the lowest MOI (0.02) in 1-day-old cells. It is noteworthy that this level of invasion is about 50% of the level observed with INT407 cells. C. jejuni entered 7- or 15-day-old differentiated Caco-2 cells at an efficiency that was even lower. The invasion efficiency decreased gradually, but not markedly, at higher MOIs, and eventually the efficiencies were equal for INT407 and Caco-2 cells (Fig. 1A).

Figure 1B shows the average number of internalized bacteria per cell, which was calculated by dividing the number of internalized bacteria by the total number of host cells per well at each MOI tested. It is noteworthy that not all host cells were infected, as discussed below. With undifferentiated host cells (INT407 cells or 1-day-old Caco-2 cells), the highest number of internalized bacteria was approximately 1.5 to 2.0 bacteria per host cell, which was observed only at MOIs of >100. Fewer bacteria entered differentiated 7- or 15-day-old Caco-2 cells (average, ~0.3 bacterium/cell), and the levels obtained required MOIs of >200 (Fig. 1B).

Distribution of bacteria interacting with differentiated Caco-2 cells. Differentiated Caco-2 monolayers on coverslips were infected with C. jejuni for times ranging from 10 to 120 min. Infected monolayers were then stained by using a fluorescence assay to quantitate all cell-associated bacteria or to distinguish intracellular bacteria from extracellular bacteria. As shown in Table 1, entry of C. jejuni was observed as early as 10 min. The total number of internalized C. jejuni bacteria (Fig. 1B) and the number of infected host cells (Table 1) increased at each time point up to 2 h postinfection with either undifferentiated INT407 cells or differentiated Caco-2 cells. However, the distributions of internalized bacteria were dramatically different for INT407 and Caco-2 cells. Approximately two-thirds of INT407 cells were infected by C. jejuni after 2 h, and each infected host cell contained about two internalized C. jejuni bacteria. In contrast, only ~20% of differentiated Caco-2 cells were infected after 2 h, and ~10% of the infected Caco-2 cells (~2% of all host cells) appeared to be highly invasion susceptible and contained 7 to 20 internalized bacteria. Notably, the internalized bacteria were distributed unevenly in differentiated Caco-2 cells, compared with the more even distribution of two internalized C. jejuni bacteria per invasion-susceptible INT407 cell. Also, a much smaller percentage of differentiated Caco-2 cells than of INT407 cells were susceptible to infection after 2 h.

When the total bacteria associated with host cells over 2 h were analyzed, ~80% of INT407 cells contained surface-bound C. jejuni 81-176 bacteria (data not shown), but only 15

temperature in 95% ethanol. Each specimen was placed in a wire mesh basket and dropped into a small beaker containing absolute ethanol. After dehydration using three additional 10-min changes of absolute ethanol, each specimen was immersed in liquid carbon dioxide and dried using a Denton DCP-1 critical point dryer. Each specimen was mounted on a stub, coated with gold-palladium using a Denton vacuum evaporator, and examined using a Hitachi S4700 field emission dryer. Each specimen was mounted on a stub, coated with gold-palladium using a Hitachi S4700 field emission dryer. Each specimen was placed in a wire mesh basket and incubated for 10 min. The total number of internalized bacteria per well and as the average number of internalized bacteria per host cell.

FIG. 1. Comparative invasion kinetics for different C. jejuni 81-176 MOIs with semiconfluent 1-day-old INT407 or Caco-2 cells and differentiated 7- or 15-day-old Caco-2 cells. Invasion assays were performed as described in Materials and Methods by testing a range of starting bacterial concentrations expressed as MOIs (numbers of bacteria added per epithelial cell). All assays were performed in duplicate and were repeated at least three times. (A) Efficiency of invasion of C. jejuni 81-176 into different host cells. The level of internalized bacteria is expressed as the percentage of the inoculum recovered after a 2-h invasion period. (B) Number of C. jejuni 81-176 bacteria internalized over a 2-h invasion period, expressed as the total number of internalized bacteria per well and as the average number of internalized bacteria per host cell.

For transmission EM, infected monolayers were grown as described above on Transwell filters. Monolayers infected with C. jejuni 81-176 for different time periods were washed three times with PBS and then fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 h at 4°C. Fixed, infected monolayers were embedded, sectioned, stained, and observed by transmission EM as previously described (23).
Undifferentiated 1-day-old Caco-2 cells

Scanning EM was used to examine the early interaction of C. jejuni with Caco-2 cells early in infection. Scanning EM was used to examine the early interaction and binding of C. jejuni at the surface of differentiated host cells. Caco-2 cells that were grown on porous Transwell membranes for 1 week formed confluent differentiated monolayers, which was confirmed by the presence of dense apical microvilli and tight intercellular junctions, as determined by high TER (see Materials and Methods).

These monolayers were infected, without centrifugation of bacteria onto the monolayers, with C. jejuni at an MOI of 10 to 100; the higher MOI made it easier to observe bound bacteria by scanning EM, but the flagellar interactions appeared to be identical at all MOIs. By 10 min postinfection, scanning EM revealed a striking interaction between the lateral surface of C. jejuni 81-176 flagella and the microvillus tips of Caco-2 cells (Fig. 2A). Figure 2B shows two C. jejuni 81-176 bacteria tethered by “torsional” binding of polar flagella to the tips of different host cell microvilli. Flagellar lateral surfaces appeared to wrap around the villus tip, a “torsional” adherence event that resulted in flagella appearing bent at angles sometimes exceeding 90°. Equivalent monolayers examined by transmission EM exhibited additional intimate contact between the bacterial outer membrane and/or surface polysaccharide and the sides or tips of Caco-2 cell microvilli (Fig. 2C and 2D). Figure 2D shows a single spiral Campylobacter bacterium (sectioned so that it appears almost like two bacteria) bound laterally to microvilli and located, as observed more than 80% of the time, near a host intercellular junctional space. To determine whether other C. jejuni strains bind Caco-2 cells via flagellar contacts, strain NCTC 11168 adherence was examined. As shown in Fig. 2E, NCTC 11168 exhibited flagellar binding with the tips and sides of microvilli, similar to the flagellar adherence observed with strain 81-176.

Events involved in C. jejuni internalization into host cells. At 10 min after infection, C. jejuni bacteria were observed to

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of host cells with associated bacteria</th>
<th>No. of internalized bacteria/infected cell (range)</th>
<th>% of infected cells with 1 to 5 internalized bacteria</th>
<th>% of infected cells with ≥7 internalized bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undifferentiated 1-day-old INT407 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>81-176</td>
<td>28.02 ± 3.48</td>
<td>1–3</td>
<td>77</td>
<td>20</td>
</tr>
<tr>
<td>NCTC 11168</td>
<td>13.36 ± 1.04</td>
<td>1–3</td>
<td>81</td>
<td>17</td>
</tr>
<tr>
<td>RY213</td>
<td>1.34 ± 0.30</td>
<td>≥1</td>
<td>100</td>
<td>0</td>
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<tr>
<td>Undifferentiated 1-day-old Caco-2 cells</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>81-176</td>
<td>19.76 ± 1.87</td>
<td>1–10</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>NCTC 11168</td>
<td>11.56 ± 0.81</td>
<td>1–7</td>
<td>62</td>
<td>20</td>
</tr>
<tr>
<td>RY213</td>
<td>1.94 ± 0.39</td>
<td>1–3</td>
<td>77</td>
<td>12</td>
</tr>
<tr>
<td>Differentiated 7-day-old Caco-2 cells</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>81-176</td>
<td>11.39 ± 0.69</td>
<td>1–5</td>
<td>59</td>
<td>23</td>
</tr>
<tr>
<td>NCTC 11168</td>
<td>16.82 ± 1.38</td>
<td>1–5</td>
<td>56</td>
<td>22</td>
</tr>
<tr>
<td>RY213</td>
<td>0.92 ± 0.18</td>
<td>≥1</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
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* Adherence assays were performed using an MOI of ~10. Caco-2 and INT407 cells were cultured for 1 to 7 days as described in Materials and Methods.
bind via flagella to Caco-2 cell microvilli (Fig. 2). These events often occurred near a tight junction. Although the infected monolayers were washed thoroughly three times in PBS before fixatives were added, we occasionally observed Campylobacter bound perpendicularly to the host cell surface. Because of the considerable washing during preparation, we believe that this event was not happenstance and reflected specific binding at one pole of the bacterium to the plasma membrane (Fig. 3A). We presumed that the polar flagella may have been bent out of the plane of sectioning and that only the proximal tips of the flagella could be seen (Fig. 3A). Figure 3B shows several bacteria apparently in different stages of the internalization process. These bacteria are associated with an irregularly shaped membrane protrusion, which may represent shortened, coalesced microvilli at the Caco-2 cell surface. Several bacteria appear to be bound to microvilli or vestigial microvilli. One bacterium has apparently just been internalized and is in an endosome, as verified recently (51). At least two bacteria appear to have been internalized previously and are in endosomes which have moved to the base of a membrane protrusion. The membrane protrusions appear to be activated sites for the uptake of multiple Campylobacter bacteria. Transmission EM examination at 60 min revealed an occasional bacterium in the junctional space (Fig. 3C), while another bacterium was adjacent to this space but appeared to be transitioning from or to the junction.

At 1 h postinfection, Campylobacter 81-176 bacteria were still attached via flagellar contacts to microvilli, but the microvilli were thicker and shorter and many of the microvilli had collapsed, leaving smooth areas visible on the host cell surface (Fig. 2F). Some bacteria appeared to be engulfed by invagination of the plasma membrane (Fig. 2F). A highly enlarged transmission EM micrograph of Campylobacter nCTC 11168 revealed the previously unobserved, convoluted, brainlike, spiral surface of Campylobacter that may play a role in interaction with host cells (Fig. 2G).

In contrast to the observations made with Caco-2 infected cells, EM of INT407 cells during the initiation of Campylobacter invasion revealed a similar, but smaller, host cell membrane extension, which was about the size of Campylobacter. Thus, Campylobacter cells interacted at the INT407 cell surface and caused formation of a small membrane extension, a protrusion, or coalesced microvilli through which the Campylobacter cells were internalized by a membrane invagination event (Fig. 3D); also, there was an adjacent tight junction.

Campylobacter 81-176 translocation involves a discrete exocytosis event. By 60 min postinfection, the ultrastructural evidence revealed movement of internalized Campylobacter cells within endosomal vacuoles from the host cell apical domain to the basolateral host cell domain. Figure 4A shows three bacteria in separate endosomes, at least two of which are associated with the perinuclear region. Figure 4B shows a Caco-2 cell containing as many as 16 bacteria, and most of these bacteria were clearly within endosomes near the basolateral surface at 1 h postinfection. After moving within endosomes, first perinuclearly and then to the basolateral host surface (Fig. 4C and D), bacteria are released by an apparent exocytosis event at the basolateral host cell surface. Figure 4C shows two bacteria near the basolateral surface; one bacterium is outside the host cell but above the transwell filter surface, and one bacterium is apparently in the process of being released from a cell by exocytosis. Figure 4D is an enlarged image of the exocytosis event shown in Fig. 4C. The endosome containing Campylobacter n has presumably fused with the basolateral host cell membrane, creating a pore through which the bacterium is being released to the extracellular space, completing a transcellular transcytosis process. This exocytosis event is not a simple reversal of the endocytosis mechanism shown in Fig. 3B and presumably occurs by a separate, unique process. However, although we consider it less likely based on our chronological studies, we cannot rule out the possibility that this event represented basolateral endocytosis of bacteria that entered paracellularly.

**DISCUSSION**

The current report describes an extension of our previous studies aimed at characterizing Campylobacter 81-176 invasion. The kinetics of Campylobacter 81-176 entry into differentiated Caco-2 cells showed a pattern with increasing MOIs similar to the pattern observed for INT407 cells, but the overall level of bacterial uptake was lower (Fig. 1). The invasion efficiency was highest at the lowest MOI (0.02), suggesting that single bacteria can trigger their own entry (29). Bacterial internalization into 7- or 15-day-old differentiated Caco-2 cells was reduced most notably (Fig. 1B). Bacterium-host cell association assays revealed that 80% of INT407 cells contained adherent bacteria but that only 15 to 20% of differentiated Caco-2 cells contained bound bacteria. For example, entry into 1-day-old INT407 cells occurred as early as 10 min and increased over 2 h, after which 68% of the host cells were consistently infected with two internalized bacteria per host cell (Table 1). In striking contrast, only 20% of polarized Caco-2 cells had internalized Campylobacter after 2 h, and the number of internalized bacteria per host cell varied from 1 to 20. Thus, far fewer

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**FIG. 2.** Scanning and transmission EM images of Campylobacter adherence to Caco-2 monolayers. (A) Scanning EM showing Campylobacter 81-176 bound via flagella to tips of Caco-2 cell microvilli (10 min postinfection; MOI, 100). Bar = 1.5 μm. (B) Enlargement of panel A, showing the lateral surfaces of flagella binding torsionally to tips of host cell microvilli (arrows). (C) Transmission EM showing cross-section of Campylobacter 81-176 exhibiting intimate bacterial surface contact (arrows) with sides and tips of microvilli (MV) (10 min postinfection). Bar = 0.2 μm. (D) Transmission EM of a longitudinal section of a single Campylobacter 81-176 cell adhering to a microvillus of a host cell (arrow) at 10 min postinfection. Thin sectioning eliminated the middle of the spiral bacterium. Note that the interaction occurs close to a tight junction (TJ). Bar = 0.5 μm. (E) Scanning EM of Campylobacter nCTC 11168 showing flagella binding to tips of Caco-2 cell microvilli (60 min postinfection; MOI, 10). Bar = 1.0 μm. (F) Scanning EM showing Campylobacter 81-176 flagella interacting with shortened microvilli and an adjacent host cell surface devoid of microvilli. The arrows indicate bacteria that are apparently being internalized by host membrane invagination (60 min postinfection; MOI, 10). Bar = 1.0 μm. (G) Enlarged scanning EM micrograph of Campylobacter nCTC 11168 showing the brainlike, convoluted surface of this spiral bacterium, which is bound via flagellar contacts to host cell microvilli. Bar = 0.5 μm.
differentiated Caco-2 cells than INT407 cells were infected over a 2-h period, suggesting that host “invasion receptor” availability may be decreased following differentiation. We speculate that this could be due to basolateral sequestering of receptors. In contrast to the even distribution of internalized C. jejuni bacteria in infected undifferentiated INT407 cells, the distribution in differentiated Caco-2 cells was uneven, and ~2% of Caco-2 cells contained 7 to 20 intracellular bacteria per cell, indicating that a small percentage of differentiated cells are hypersusceptible to Campylobacter invasion. These cells may represent M-like cells, the domes of which have been observed with differentiated Caco-2 cells and which collapse during C. jejuni infection (33). Recently, coculture of Caco-2 cells with Raji B lymphocytes has been shown to result in the differentiation of M-like cells, defined by expression of surface galectin-9 (42). Unlike the situation for mouse M cells, there

FIG. 3. Transmission EM micrographs showing apparent stages in C. jejuni 81-176 invasion of Caco-2 and INT407 host cells. (A) C. jejuni interacting end-first with a bald (microvillus-free) host cell apical surface, apparently penetrating the host cell with its polar flagellum (10 min postinfection; MOI, ~100). The proximal end of each polar flagellum is indicated by an arrow; thin sectioning apparently removed most of the flagella. A nearby tight junction (TJ) at the bottom of the micrograph is indicated by an arrow. Bar = 0.5 μm. (B) Bacteria associated with an irregularly shaped, “invasion-activated” membrane protrusion of the Caco-2 apical cell surface. Three bacteria (asterisks) are interacting with apparent vestigial microvilli, one C. jejuni cell has presumably just been engulfed by endocytosis (arrow), and two bacteria were apparently previously internalized (arrowheads) and have moved to the base of the membrane protrusion (10 min postinfection). Bar = 0.5 μm. (C) At later times after infection, C. jejuni bacteria (arrow) are occasionally found in junctional spaces (TJ, tight junction); these bacteria may have entered paracellularly, followed by junctional readherence, or could have exited the host cell laterally after they were first endocytosed (60 min postinfection). Bar = 1 μm. (D) Small “invasion-activated” membrane protrusion of an INT407 host cell through which a C. jejuni bacterium (arrow) has apparently just been internalized. This event occurred immediately adjacent to a cellular tight junction (10 min postinfection; MOI, ~100). Bar = 0.2 μm.
are no commercially available antibodies to identify human M cells, which prevented confirmation of this interesting possibility. Both scanning EM and transmission EM were used to examine the early \textit{Campylobacter}–Caco-2 cell interactions in differentiated monolayers grown on Transwell filters. Scanning EM revealed unique torsional contacts between the sides of flagella and microvillus tips, in contrast to previous reports of flagellar tip adherence (26, 28), possibly due to the higher-resolution images obtained in the current study. Both \textit{C. jejuni} 81-176 and NCTC 11168 were typically tethered at the host surface by each polar flagellum binding laterally to different microvillus tips (Fig. 2 A, B, E, and G), an interaction which suggests that there is specific binding between the sides of flagella and components of the rounded tips of microvilli. Transmission EM revealed additional contacts between the bacterial cell surface and the sides of microvilli (Fig. 2C and D). The latter contacts may involve capsular polysaccharide, Peh1, JlpA, or other outer membrane proteins which have been reported to be involved in adherence (2, 24, 39–41). \textit{C. jejuni} has a brainlike convoluted surface (Fig. 2G), which may play a role in host cell-bacterium interactions. We speculate that ligands located at the apical surface (instead of deep in the folds) of the convolutions may be more active in host cell interactions. The bacterium-host cell interactions described above were readily observed early and throughout the invasion period. We presume that they represent different types of adherence, some of which may be reversible.

![Image](http://iai.asm.org/.../CJ.jpg)
Flagella have long been implicated as Campylobacter adhesins (34, 37). Flagellum-dependent motility and the flagellum itself appear to be essential for C. jejuni invasion of epithelial cells, but the mechanism(s) remains unknown (1, 12, 50, 52, 53). It seems unlikely that the Campylobacter flagellar adherence is casual because the infected monolayers were washed six times with Earle’s balanced salt solution prior to fixation for scanning EM, a process that involves an additional 10 washes and buffer changes. Although most EM studies were performed with an MOI of 100, using an MOI of 10 resulted in similar bacteria-host cell interactions, but the frequencies were much reduced. We speculate that C. jejuni flagellum-host cell interactions represent a primary adherence mechanism that augments the contact of “invasion-specific” bacterial ligands with host membrane receptors in lipid rafts, which result in signal transduction events that lead to bacterial internalization. Since ~10% of the invasion ability can be restored to nonflagellated C. jejuni cells by centrifugation onto a monolayer (50), we suggest that “invasion-specific” C. jejuni ligands may be located at the bacterial poles, which are normally adjacent to the polar flagella in wild-type C. jejuni. Strain NCTC 11168 is known to be less invasive than 81-176, possibly due to reduced numbers of “invasion-specific” ligands or expression of surface factors that interfere with these ligands.

C. jejuni cells were observed to interact typically with microvilli at the apical Caco-2 cell surface adjacent to intercellular junctions, as noted previously (28). Very infrequently, Campylobacter bacteria were observed bound perpendicular to the host cell, possibly with one polar flagellum interacting with the host cell (Fig. 3A); a similar observation of perpendicular bacterial interaction was recently reported (31). This is a relatively difficult event to observe, possibly because the organism is internalized rapidly, and it is technically difficult to find a thin section containing such a physically space-limited event. Figure 3A shows that the local host membrane surface lacks the typical dense microvilli shown in Fig. 2D, and this may reflect a differentiated M-like cell surface. We suggest that intimate bacterium-host cell contact, as shown in Fig. 3A, B, or D, may be “invasion specific” and that binding of bacterial ligands with specific host plasma membrane receptors may have triggered a localized collapse of the terminal F-actin web, leading to shortening and coalescence of microvilli. Previously, we and other workers have reported that pretreatment of monolayers with cytochalasin D actually enhances Campylobacter invasion (19, 38, 51), possibly by eliminating the terminal actin web. Thus, initial actin depolymerization and subsequent polymerization are probably involved in Campylobacter entry. In fact, a recent report showed that Rac1 and Cdc42 are involved and that there may be membrane ruffling prior to C. jejuni invasion (31). We have no evidence of effector secretion into host cells via the bacterium-host cell interaction shown in Fig. 3A, but we cannot exclude this interesting possibility. Konkel and coworkers (27) suggested that effector secretion is important in Campylobacter invasion.

An early membrane dynamic event observed during Campylobacter entry is the formation of an activated membrane protrusion, which may represent a coalescence of local microvilli. We speculate that invasion-specific bacterium-host cell contact may promote host signaling events that activate the membrane in a localized region, leading to bacterial internalization through this “activated” site (Fig. 3B). “Activation” may encompass host signaling events, as well as cytoskeletal and membrane alterations. In differentiated Caco-2 cells, this “activated” membrane protrusion is approximately the length of one bacterium wide and 2 to 3 lengths high and can apparently undergo multiple bacterial internalization events. In contrast, the comparable invasion-specific, “activated” membrane extension observed in undifferentiated INT407 cells during C. jejuni invasion is smaller and apparently engulfs a single organism (Fig. 3D). Regardless of the host membrane protrusion size, bacterial internalization apparently results from plasma membrane invagination that begins at the bacterium-host cell contact site (19, 26, 29, 31). As observed previously (28, 38), the bacteria are internalized into an endosome, which is transported over time from the apical host surface to the basolateral host surface (Fig. 4). Our previous confocal microscopic analyses suggested that C. jejuni 81-176 cells are transported in endosomes via the molecular motor dynein along MTs (19). Watson and Galan (51) recently reported that Campylobacter cells reside and transit within special endosomes that avoid fusion with lysosomes. At later infection times, an occasional bacterium is observed in the intercellular space below apical junctional adherence (Fig. 3C). This event has not been observed commonly or earlier in infection, leading us to suggest that this bacterium just exited the host cell laterally; however, the possibility of paracellular entry from the apical surface followed by junctional rescaling cannot be ruled out (8). Based on the current study, we suggest that C. jejuni bacteria in endosomes are transported basolaterally through the cell and can undergo specific endosome-basolateral membrane fusion (Fig. 4D), an exocytosis process which releases C. jejuni subepithelially. This process does not visually appear to be a simple reversal of the apical endocytic mechanism. Although we consider a basolateral endocytosis event unlikely, this possibility cannot be ruled out by the results of EM. Previous reports indicated that the ability of C. jejuni to invade Caco-2 cells is not linked to translocation ability (7, 17), also suggesting that endocytosis and exocytosis are unique mechanistic events. Based on these observations, we speculate that C. jejuni bacteria proficient in transcytosis across the epithelium may be able to alter the Campylobacter-endosome membrane so that they traffic through the cell and can fuse with the host plasma membrane, resulting in basolateral exocytosis. Since monolayer TER is decreased at later infection times (33) and alterations in tight junction proteins have been observed at 24 to 48 h postinfection (8, 33), it seems reasonable to suggest that intercellular junction damage may occur at later times, following C. jejuni transcytosis across the intestinal epithelium.

In summary, this study revealed new specific C. jejuni-host cell interactions and structures involved in adherence to, invasion of, and translocation across differentiated Caco-2 cells that were not observed in previous studies. C. jejuni interactions with differentiated Caco-2 cells likely are more representative of events that actually occur in the intestine; nevertheless, INT407 cells have provided much useful information on C. jejuni adherence and invasion mechanisms. In contrast to the relatively consistent level of 2 internalized bacteria per undifferentiated INT407 cell, certain differentiated Caco-2 cells appeared to be hypersusceptible to invasion and contained as many as 20 internalized C. jejuni
bacteria. Whether these hypersusceptible Caco-2 cells are human M-like cells could not be verified due to a lack of available reagents. Campylobacter transcytosis appears to involve discrete endocytic and exocytic events not previously observed by transmission EM. Together, these findings provide an improved concept for C. jejuni 81-176 adherence, endocytosis, and exocytosis mechanisms and indicate potentially important processes and structures that should be characterized further.

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REFERENCES

32. Sansonetti, P. J., and A. Phalipon. 1999. M cells as ports of entry for...


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