

Increase in *Campylobacter jejuni* Invasion of Intestinal Epithelial Cells under Low-Oxygen Coculture Conditions That Reflect the *In Vivo* Environment

Dominic C. Mills,^a Ozan Gundogdu,^a Abdi Elmi,^a Mona Bajaj-Elliott,^b Peter W. Taylor,^c Brendan W. Wren,^a and Nick Dorrell^a

Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom^a; Department of Infectious Diseases and Microbiology, Institute of Child Health, London, United Kingdom^b; and Department of Pharmaceutics, The School of Pharmacy, London, United Kingdom^c

***Campylobacter jejuni* infection often results in bloody, inflammatory diarrhea, indicating bacterial disruption and invasion of the intestinal epithelium. While *C. jejuni* infection can be reproduced *in vitro* using intestinal epithelial cell (IEC) lines, low numbers of bacteria invading IECs do not reflect these clinical symptoms. Performing *in vitro* assays under atmospheric oxygen conditions neither is optimal for microaerophilic *C. jejuni* nor reflects the low-oxygen environment of the intestinal lumen. A vertical diffusion chamber (VDC) model system creates microaerobic conditions at the apical surface and aerobic conditions at the basolateral surface of cultured IECs, producing an *in vitro* system that closely mimics *in vivo* conditions in the human intestine. Ninefold increases in interacting and 80-fold increases in intracellular *C. jejuni* 11168H wild-type strain bacteria were observed after 24-h coculture with Caco-2 IECs in VDCs under microaerobic conditions at the apical surface, compared to results under aerobic conditions. Increased bacterial interaction was matched by an enhanced and directional host innate immune response, particularly an increased basolateral secretion of the proinflammatory chemokine interleukin-8 (IL-8). Analysis of the invasive ability of a nonmotile *C. jejuni* 11168H *rpoN* mutant in the VDC model system indicates that motility is an important factor in the early stages of bacterial invasion. The first report of the use of a VDC model system for studying the interactions of an invasive bacterial pathogen with IECs demonstrates the importance of performing such experiments under conditions that represent the *in vivo* situation and will allow novel insights into *C. jejuni* pathogenic mechanisms.**

The Gram-negative, microaerophilic organism *Campylobacter jejuni* is one of the most common causes of food-borne bacterial gastroenteritis in developed countries (6), with 500,000 and 2,500,000 predicted cases each year in the United Kingdom and the United States, respectively (27). The predominant route of transmission is consumption and handling of undercooked, contaminated poultry (20). Once ingested, *C. jejuni* can lead to symptoms ranging from mild, watery diarrhea to severe, bloody inflammatory diarrhea (1). The majority of *C. jejuni* infections are self-limiting; however, infection with *C. jejuni* can potentially lead to postinfectious sequelae, such as Guillain-Barré syndrome, reactive arthritis, and inflammatory bowel disease (8, 22, 38).

Despite the prevalence of *C. jejuni* as a causative agent of gastroenteritis, knowledge of the molecular basis of pathogenesis and interactions with host cells is still very limited compared to that for other enteropathogens, such as *Salmonella* species, *Yersinia* species, *Shigella* species, and pathogenic *Escherichia coli* (47). This knowledge gap can in part be attributed to the lack of a convenient, reproducible small-animal model system to study *C. jejuni*-host interactions (15). Although several animal models have been used, each one has major drawbacks. Animal models using either ferrets (4) or rhesus monkeys (40, 41) have been shown to closely mimic the disease observed in humans. However, the facilities required for the handling of such animals, the unavailability of host genetic manipulation techniques, and the relatively long generation time render these animals impractical for regular use in most laboratories. Chickens, as a natural host of *C. jejuni*, can easily be experimentally inoculated (13). However, although both chicks and chickens have been used successfully in various studies (18, 24), such studies reflect *C. jejuni* colonization, and the direct relevance of the chick and chicken models to human campylobac-

teriosis is debatable. The model organism most frequently used to study human pathogens is the mouse. Indeed, experimental inoculation of mice with *C. jejuni* has been performed for nearly 30 years (7). However, differences in the mouse strains used, the pretreatments of the mice, the routes of inoculation, and the inoculation loads have resulted in findings as diverse as noncolonization, nonsymptomatic carriage, or severe diarrhea (3, 8, 43, 48). Knockout mouse models of *C. jejuni* enteritis using mice deficient in NF- κ B (19), MyD88 (45), interleukin-10 (IL-10) (33), and Nramp1 (9) have been reported. Additionally, infections of mice with limited enteric flora have been reported (10). The outcomes of *C. jejuni* infection in these models differ between genetically engineered mice, suggesting that a robust, reproducible, “gold standard” mouse model for *C. jejuni* infection remains elusive, and as such the *C. jejuni* research community has yet to adopt a defined mouse model for pathogenesis studies.

In the absence of a convenient, reproducible small-animal model, tissue culture assays represent a useful alternative. *C. jejuni* has been shown to adhere to and invade various polarized and nonpolarized intestinal epithelial cells (IECs) *in vitro*, including

Received 10 November 2011 Returned for modification 16 December 2011

Accepted 3 February 2012

Published ahead of print 21 February 2012

Editor: B. A. McCormick

Address correspondence to Nick Dorrell, nick.dorrell@lshtm.ac.uk.

Supplemental material for this article may be found at <http://iai.asm.org/>.

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doi:10.1128/IAI.06176-11

the Caco-2 (16), INT 407 (31), and T84 (35) cell lines. However, the reported adhesion and invasion interactions of *C. jejuni* with IECs are minimal compared to those of other enteric pathogens, with often less than 1% of the starting inoculum recovered intracellularly following gentamicin protection assays (21). This low level of adhesion and invasion does not correlate with the clinical presentation of *C. jejuni* infection in humans (21). One explanation for these low adhesion and invasion levels is that coculturing of *C. jejuni* with IECs is routinely performed under atmospheric oxygen conditions, as this is required for survival of the IECs. Even though the microaerophilic *C. jejuni* possesses several defense mechanisms against oxidative stress, such as the SodB superoxide dismutase (39) and the KatA catalase (14), it is likely that the bacterium behaves differently under atmospheric oxygen conditions than in the natural low-oxygen environment of the intestinal lumen.

When the microaerophilic human pathogen *Helicobacter pylori* was cocultured with epithelial cells with microaerobic conditions at the apical surface and aerobic conditions at the basolateral surface using a vertical diffusion chamber (VDC), or Ussing chamber, the result was a significant increase in bacterial adherence under microaerobic conditions compared to the level under aerobic conditions (12). An increase in the expression of the *H. pylori* virulence factor CagA and changes in the host response were also observed (12). However, the VDC system has not previously been used to study an invasive enteric bacterial pathogen. The use of a similar VDC model system for *C. jejuni* infection of IECs will mimic the *in vivo* situation more closely than other tissue culture assays and as such should allow more-accurate investigations of host-pathogen interactions. In this study, a modified VDC system was used to allow the coculture of *C. jejuni* with IECs under microaerobic conditions in the apical compartment, resulting in an 80-fold increase in levels of bacterial invasion and an enhanced host innate immune response.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* wild-type strains used in this study were 11168H and 81-176. 11168H is a genetically stable hypermotile derivative (28, 30) of the original sequenced strain NCTC11168 (37). 11168H shows much higher colonization levels than the NCTC11168 strain in a chick colonization model (28) and is thus considered a better strain to use for host-pathogen interaction studies. 81-176 is a gastroenteritis isolate from a multistate outbreak from contaminated milk, widely used for *C. jejuni* infection and human volunteer studies (5). The *C. jejuni* 11168H *rpoN* mutant was obtained from the LSHTM *Campylobacter* Resource Facility (<http://crf.lshtm.ac.uk/index.htm>). *C. jejuni* strains were routinely cultured on blood agar (BA) plates supplemented with *Campylobacter* selective supplement (Oxoid, Basingstoke, United Kingdom) and 7% (vol/vol) horse blood (TCS Microbiology, Botolph Claydon, United Kingdom) at 37°C in a variable atmosphere incubator (VAIN) microaerobic chamber (Don Whitley Scientific, Sheffield, United Kingdom) containing 85% N₂, 10% CO₂, and 5% O₂. Appropriate antibiotics were added as follows: ampicillin (100 µg/ml), kanamycin (50 µg/ml), and chloramphenicol (50 µg/ml for *E. coli* studies or 10 µg/ml for *C. jejuni* studies). All reagents were obtained from Invitrogen (Paisley, United Kingdom) unless otherwise stated.

Epithelial cell line and culture conditions. The human Caco-2 IEC line was cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS; Sigma-Aldrich, Poole, United Kingdom), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% (vol/vol) nonessential amino acids and maintained at 37°C in 5% CO₂ and 95% air. The human T84 IEC line was cultured in 1:1 DMEM-F-12 medium (Invitrogen) supplemented with 10% (vol/vol) FCS and 100

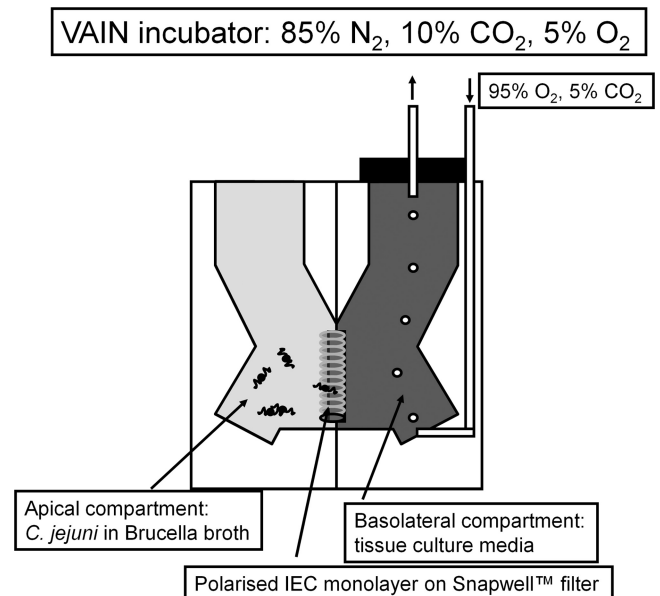


FIG 1 Vertical diffusion chamber (VDC) model system. IECs were grown to polarization on Snapwell filters and placed between the two compartments of the VDC. The apical compartment was filled with brucella broth, and the basolateral compartment was filled with tissue culture medium. For infection studies, *C. jejuni* was added to the apical compartment of the VDC. For the aerobic controls, the VDC was left open and placed into a tissue culture incubator containing air enriched with 5% CO₂. For microaerobic coculturing, the VDC was placed into a microaerobic incubator with the apical compartment open to the atmosphere within the incubator and the basolateral compartment closed and perfused with 95% O₂ and 5% CO₂.

U/ml penicillin, 100 µg/ml streptomycin. For VDC experiments, 4×10^5 Caco-2 or T84 IECs were seeded into the upper compartment of a Snapwell filter (Corning Lifesciences, Amsterdam, Netherlands). To allow for the formation of a polarized monolayer, cells were grown for a minimum of 21 days for Caco-2 IECs or 14 days for T84 IECs. The growth medium was changed every 3 days.

Assembly of the vertical diffusion chamber model system. Prior to assembly, the two compartments of the VDC (Harvard Apparatus, Holliston, MA) were sterilized by immersion in Haz-Tabs (Guest Medical, Ltd., Aylesford, United Kingdom) solution for 3 h, followed by three washes with sterile water. A Snapwell filter carrying a polarized monolayer of Caco-2 or T84 IECs was removed from the culture plate, washed three times with phosphate-buffered saline (PBS), and inserted between the two compartments of the VDC. The basolateral compartment was filled with 4 ml of the appropriate tissue culture medium supplemented with 1% (vol/vol) FCS and 1% (vol/vol) nonessential amino acids. The apical compartment was filled with 4 ml brucella broth (Oxoid). For infections, approximately 4×10^8 *C. jejuni* cells were harvested from a 24-h BA plate and added to the apical compartment. For aerobic coculturing, the VDC was maintained at 37°C in 5% CO₂ and 95% air. For microaerobic coculturing, the VDC was maintained under microaerobic conditions (85% N₂, 10% CO₂, and 5% O₂) in a VAIN. A gas mixture of 95% O₂ and 5% CO₂ was perfused through the basolateral compartment, while the apical compartment was left open to the microaerobic atmosphere in the VAIN (Fig. 1).

Immunofluorescence analysis of cellular distribution of actin and occludin. IECs were fixed with 2% (wt/vol) paraformaldehyde (Sigma-Aldrich) for 1 h at 4°C, permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 20 min, and then blocked with 1% (wt/vol) bovine serum albumin (BSA) in PBS for 1 h, both at room temperature. The filter was excised from the carrier and placed in a 12-well dish. For actin staining, IECs were

incubated with Alexa Fluor 555-conjugated phalloidin (Invitrogen) (stock diluted 1:1,000 in PBS) for 1 h in the dark. For occludin staining, IECs were incubated with mouse antioccludin primary antibody (stock diluted 1:100 in PBS) (Invitrogen) for 1 h at room temperature, followed by Alexa Fluor 488-conjugated goat anti-mouse (Invitrogen) (stock diluted 1:200 in PBS) for 1 h at room temperature in the dark. Stained filters were mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; final concentration, 1.5 $\mu\text{g}/\text{ml}$) (Vector Laboratories, Peterborough, United Kingdom) on a coverslip (Fisher Scientific, Loughborough, United Kingdom) and examined with a Zeiss LSM510 confocal microscope (Carl Zeiss AG, Jena, Germany).

Fluorescent dextran diffusion assay. IECs on Snapwell filters were washed three times with sterile PBS and placed back into the hanging support; 500 μl of 100 μM fluorescein isothiocyanate (FITC)-labeled dextran (Sigma-Aldrich) with an average molecular mass of 4 kDa in Ringer's solution (115 mM NaCl, 1 mM KCl, 1 mM CaCl_2) was added to the apical side of the monolayer and incubated for 3 h at room temperature, with the basolateral side of the monolayer immersed in Ringer's solution. The amount of fluorescently labeled dextran on the basolateral side of the monolayer was determined postincubation by removal of the basolateral solution and measurement of the fluorescence intensity at 488 nm using a Gemini XPS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of Caco-2 monolayer TEER in the VDC. The transepithelial electrical resistance (TEER) of a Caco-2 monolayer in a VDC was measured by placing two voltage-sensing AgCl electrodes close to the cell monolayer on each side of the insert, passing a current through two further electrodes placed at the two distal ends of the VDC, and reading the voltage necessary to keep the current flowing. Resistance was calculated according to Ohm's law ($R = V/I$, where R = resistance, V = voltage, and I = current) and multiplied by the surface area of the monolayers (1.12 cm^2).

Enumeration of interacting bacteria and intracellular bacteria. At the desired time point of coculturing, the apical and basolateral supernatants were removed from the VDC and stored at -80°C for subsequent analysis. The Snapwell filter was removed from the VDC, washed three times with sterile PBS, and placed into a 6-well tissue culture dish. IECs were lysed by addition of 0.1% (vol/vol) Triton X-100 in PBS for 20 min at room temperature. The lysates were serially diluted in PBS, plated on BA plates, and incubated microaerobically for 72 h. CFU counts were determined, and the number of bacteria interacting with the IECs was calculated. Enumeration of intracellular bacteria was performed essentially as described above, with the following modification. Before lysis with Triton X-100, the IECs were incubated in DMEM containing 150 $\mu\text{g}/\text{ml}$ gentamicin (Sigma-Aldrich) for 2 h at 37°C . This step kills extracellular, adherent bacteria and allows for the analysis of the number of intracellular bacteria present after coculturing. All VDC experiments were performed with at least two technical replicates and at least three biological replicates per experimental data set.

Cytokine analysis of the coculturing supernatants. Coculturing supernatants were probed for the presence of interleukin-8 (IL-8) with a human IL-8 enzyme-linked immunosorbent assay (ELISA) development kit (Peprotech, London, United Kingdom), according to the manufacturers' instructions.

Microarray analysis of *C. jejuni* 11168H gene expression profiles. Gene expression profiles of *C. jejuni* 11168H in the apical compartment of the VDC after coculture with Caco-2 IECs for 6 h or 24 h under either aerobic or microaerobic conditions were analyzed using an indirect-comparison method or type 2 experimental design (46). Replicate test sets of Cy5-labeled *C. jejuni* 11168H total RNA samples were combined with a common reference sample (Cy3-labeled *C. jejuni* 11168H genomic DNA) using methodology described previously (23). Whole-genome *C. jejuni* NCTC11168 microarrays printed on UltraGAPS glass slides (Corning Lifesciences), constructed by the B μ G@S microarray group (<http://www.bugs.sgul.ac.uk/>), were used in this study (29). The microarray slides were scanned with an Affymetrix 418 array scanner (MWG Biotech, Ebersberg,

Germany) according to the manufacturer's guidelines. Signal and local background intensity readings for each spot were quantified using ImaGene software v8.0 (BioDiscovery, El Segundo, CA). Quantified data were analyzed using GeneSpring GX software v7.3 (Agilent, Santa Clara, CA). Statistically significantly up- and downregulated genes were selected by comparing 11168H gene expression under microaerobic conditions against that under aerobic conditions using analysis of variance (ANOVA) with a Benjamini and Hochberg false-discovery rate as the multiple testing correction (2, 11).

Complementation of the *C. jejuni* 11168H *rpoN* mutant. Complementation was performed by inserting a copy of the *rpoN* gene into the *rpoN* mutant chromosome with a *C. jejuni* NCTC11168 complementation vector (25), using previously described techniques (23). The complementation vector utilizes the constitutive chloramphenicol cassette promoter to express the *rpoN* gene and not the native *rpoN* promoter. The coding region for *rpoN* was amplified by PCR using proofreading *Pfu* polymerase (Fermentas, Sankt Leon-Rot, Germany) and ligated into the NcoI and NheI sites on the complementation vector. This construct was checked by sequencing (data not shown) and electroporated into the 11168H *rpoN* mutant. Putative clones were selected on BA plates containing kanamycin and chloramphenicol. Confirmation of the presence of copies of both *rpoN* and *rpoN* Km^r was performed by PCR and also by sequencing (data not shown).

Statistical analysis. Data were statistically analyzed using Prism software (GraphPad Software, Inc., La Jolla, CA). Figures display means \pm standard errors as well as the P values determined by unpaired Student t tests. All experiments represent at least three biological replicates performed in triplicate in each experiment.

Microarray data accession numbers. The array design is available in B μ G@Sbase (accession no. A-BUGS-9; <http://bugs.sgul.ac.uk/A-BUGS-9>) and also ArrayExpress (accession no. A-BUGS-9). Fully annotated microarray data have been deposited in B μ G@Sbase (accession no. E-BUGS-125; <http://bugs.sgul.ac.uk/E-BUGS-125>) and also ArrayExpress (accession no. E-BUGS-125).

RESULTS

Cellular distribution of actin and occludin within IECs is not affected by culture in a VDC with microaerobic conditions at the apical surface. Prior to using the VDC to coculture *C. jejuni* and IECs with microaerobic conditions at the apical surface, the effect of these conditions on polarized IECs was determined. The morphology of Caco-2 monolayers after 24 h of incubation in a VDC was analyzed by confocal laser microscopy. No difference in distribution of actin between IECs that had been maintained under microaerobic conditions (Fig. 2B) or under aerobic conditions (Fig. 2A) was detected. The fluorescence signal localized predominantly to the junctions between adjacent cells. Additionally, a strong localization of actin to the apical side of the Caco-2 IECs was noted, indicating the formation of a dense microvillus brush border. This indicated that the IECs were not adversely affected under microaerobic conditions and remained attached to the Snapwell filter. As the distribution of actin does not provide any information on the intactness of monolayers, the presence of intact tight junctions between the IECs was also analyzed. No difference in distribution of occludin between IECs that had been maintained under microaerobic conditions (Fig. 2B) or under aerobic conditions (Fig. 2A) was detected. Fluorescence was tightly localized to the cell-cell boundaries, indicating the presence of intact tight junctions. A similar distribution of actin and occludin was observed with T84 IECs (data not shown).

The barrier function of polarized IECs is not affected by culture in a VDC with microaerobic conditions at the apical surface. To quantitatively assess monolayer integrity after culture in a

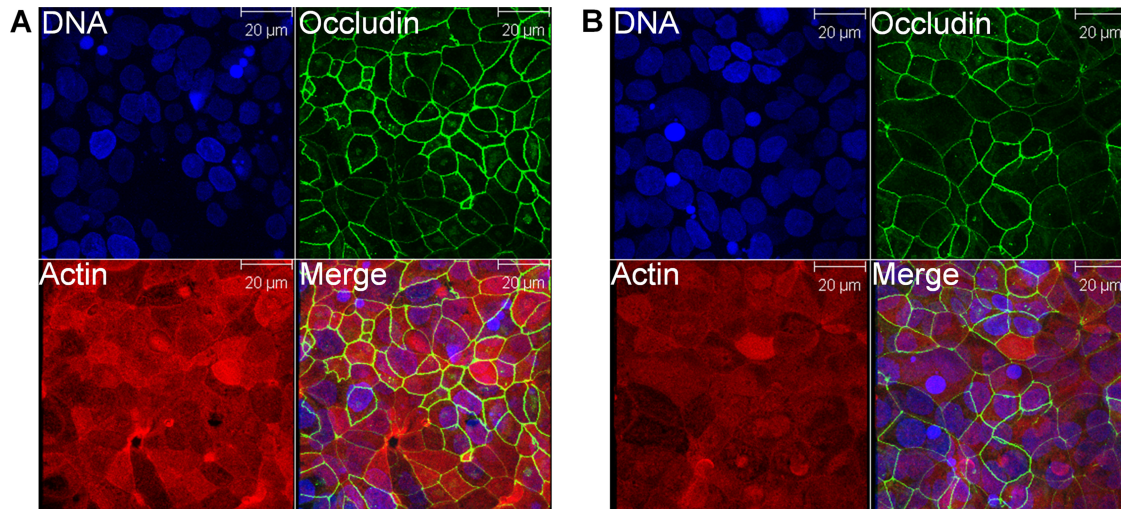


FIG 2 Microaerobic conditions and bacterial broth on the apical side of intestinal epithelial cells (IECs) in a vertical diffusion chamber (VDC) have no detrimental effect on Caco-2 IECs over 24 h. Caco-2 IECs were grown for 21 days on Snapwell filters and maintained in VDCs with brucella broth and either aerobic (A) or microaerobic (B) conditions in the apical compartment for 24 h. After the incubation, the IECs were processed for immunostaining and stained for occludin (green) and actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

VDC, the diffusion of fluorescently labeled dextran across Caco-2 (Fig. 3A) and T84 (Fig. 3B) monolayers was determined. Approximately 25% of the labeled dextran diffused across an empty Snapwell filter after 3 h. However, there was no significant difference in dextran diffusion between IECs grown on Snapwell filters and

IECs maintained in a VDC for 24 h with either microaerobic or aerobic conditions at the apical surface. Dextran diffusion was dramatically increased across IECs grown on Snapwell filters after permeabilization with Triton X-100, demonstrating that it is the barrier function of the polarized IECs that prevents dextran diffu-

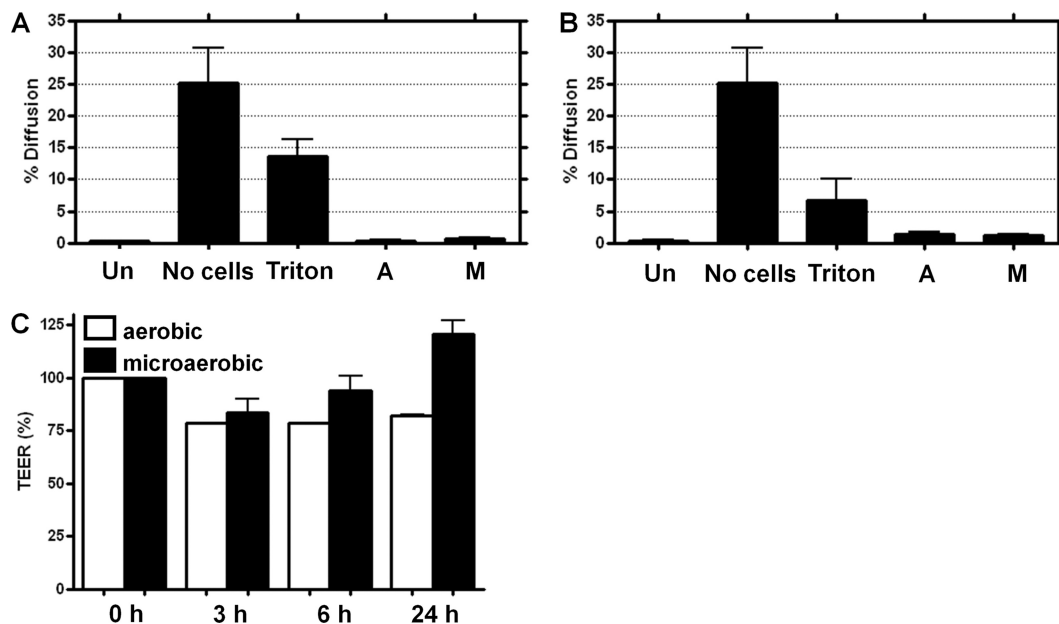


FIG 3 Analysis of the diffusion of a fluorescent marker across cellular monolayers on Snapwell filters following incubation in the vertical diffusion chamber (VDC). Caco-2 (A and C) or T84 (B) intestinal epithelial cells (IECs) were grown for 21 or 14 days, respectively, on Snapwell filters and maintained in VDCs with brucella broth and either aerobic (A) or microaerobic (M) conditions in the apical compartment for 24 h. VDCs were dismantled; 500 μ l of 100 μ M FITC-labeled dextran (average molecular mass of 4 kDa) in Ringer's solution was added to the apical side of the monolayer, and cells were incubated for 3 h at room temperature (A and B). After 3 h, the percentage of FITC-labeled dextran that had passed across the monolayer was determined from the relative fluorescence of the basolateral solution and the relative fluorescence of the input solution. An empty Snapwell filter (No cells), IECs grown for 21 days on Snapwell filters and permeabilized with 0.5% (vol/vol) Triton X-100 for 20 min at room temperature (Triton), and IECs grown for 21 days on Snapwell filters (Un) were used as controls. The transepithelial electrical resistance (TEER) was measured after assembly of the VDCs and set as 100% (C). After 3 h, 6 h, and 24 h postassembly, the TEER was measured and calculated as a percentage of the value obtained at time point 0.

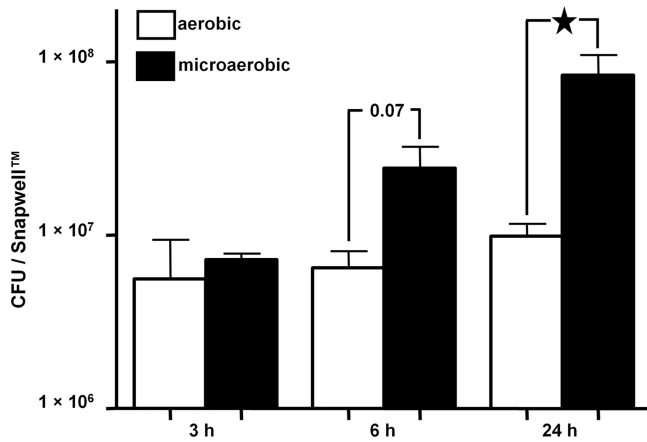


FIG 4 *C. jejuni* interactions with intestinal epithelial cells (IECs) are significantly increased under microaerobic conditions. *C. jejuni* 11168H wild-type strain bacteria were cocultured with Caco-2 IECs in a vertical diffusion chamber for 3 h, 6 h, and 24 h with either aerobic or microaerobic conditions in the apical compartment, and the numbers of interacting bacteria were assessed. ★, $P < 0.05$.

sion. Measurement of the TEER of an IEC monolayer on a permeable support is a direct, quantitative method for analysis of the polarization status of the IEC monolayer. There was no reduction in the TEER of a Caco-2 monolayer over a 24-h period under microaerobic conditions compared to the level under aerobic conditions (Fig. 3C).

***C. jejuni* interactions with IECs are enhanced during coculture in a VDC under microaerobic conditions.** The *C. jejuni* 11168H wild-type strain was cocultured with Caco-2 IECs at a multiplicity of infection (MOI) of approximately 100:1 with either microaerobic or aerobic conditions in the apical compartment for 3, 6, and 24 h (Fig. 4). The numbers of bacteria interacting with the IECs under microaerobic conditions increased markedly over this

period. After 24 h, a significant ($P < 0.05$) 9-fold increase in interacting *C. jejuni* 11168H bacteria was observed under microaerobic conditions compared to the number under aerobic conditions. It is possible that the increased numbers of interacting bacteria under microaerobic conditions may be the result of an increase in bacterial numbers during the 24-h assay due to increased proliferation of *C. jejuni* under microaerobic conditions. However, serial dilution plating of the contents of the apical compartment demonstrated equal numbers of bacteria present under microaerobic and aerobic conditions after 24 h of coculturing (data not shown). This indicated that the observed increase in *C. jejuni* interactions with IECs under microaerobic conditions was due to changes in bacterial activity, rather than an increase in bacterial numbers. Another possibility is that the microaerophilic conditions affect the activity and/or biology of the IECs, which then become more susceptible to *C. jejuni* infection.

***C. jejuni* invasion of IECs is dramatically enhanced during coculture in a VDC under microaerobic conditions.** The *C. jejuni* 11168H wild-type strain was cocultured with Caco-2 IECs at an MOI of approximately 100:1 with either microaerobic or aerobic conditions in the apical compartment for 6 and 24 h, and the numbers of intracellular bacteria were determined (Fig. 5A). There was a significant ($P < 0.05$) 5-fold increase in the numbers of intracellular *C. jejuni* bacteria after 6 h under microaerobic conditions. After 24 h, there was a significant ($P < 0.01$) 80-fold increase in intracellular *C. jejuni* bacteria recovered under microaerobic conditions. To confirm that the observed increase in numbers of intracellular *C. jejuni* bacteria after 24 h of coculturing in the VDC under microaerobic conditions was not a specific effect of the bacterial strain or IEC line used, two further experiments were performed. The *C. jejuni* 81-176 wild-type strain was cocultured for 24 h with Caco-2 IECs with either aerobic or microaerobic conditions in the apical compartment (Fig. 5B). After 24 h, there was a significant ($P < 0.05$) 89-fold increase in intracellular *C. jejuni* 81-176 bacteria recovered from Caco-2 cells un-

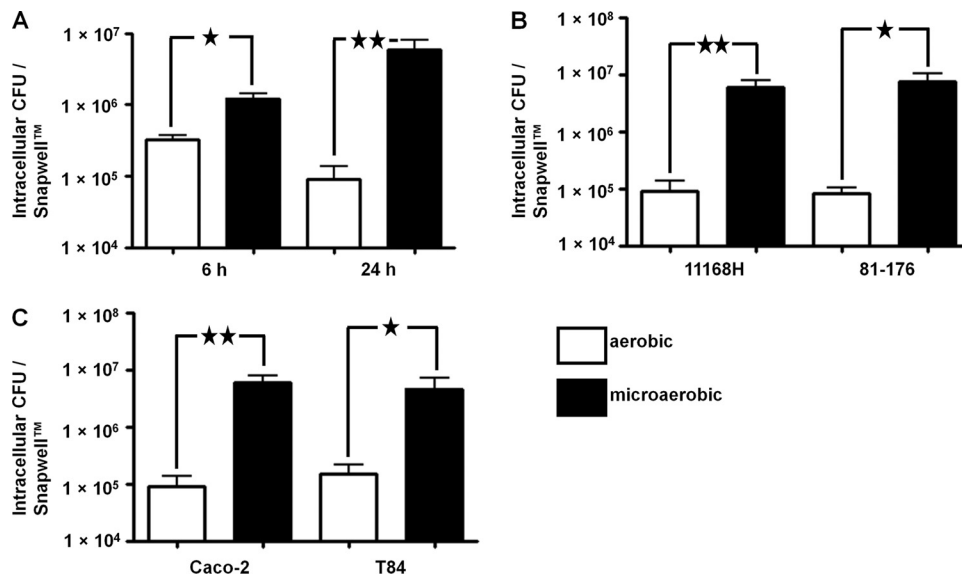


FIG 5 *C. jejuni* invasion of intestinal epithelial cells (IECs) is significantly increased under microaerobic conditions. *C. jejuni* wild-type strains were cocultured with IECs in a vertical diffusion chamber with either aerobic or microaerobic conditions in the apical compartment, and the numbers of intracellular bacteria were assessed. (A) 11168H cocultured with Caco-2 IECs for 6 h and 24 h. (B) 11168H or 81-176 cocultured with Caco-2 IECs for 24 h. (C) 11168H cocultured with Caco-2 or T84 IECs for 24 h. ★, $P < 0.05$; ★★, $P < 0.01$.

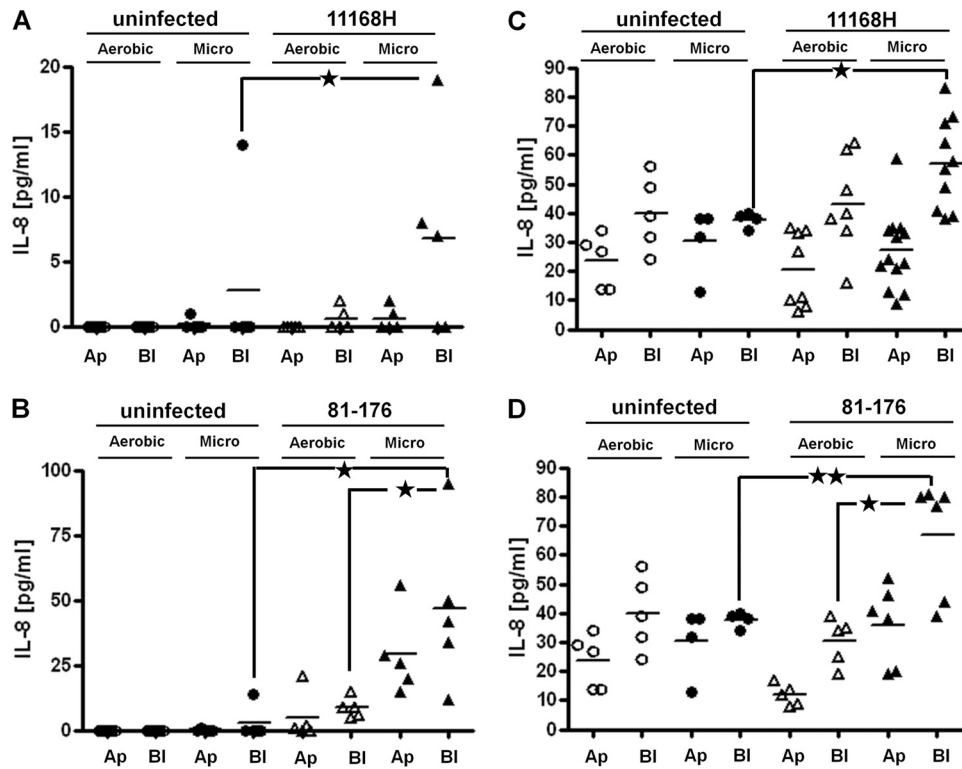


FIG 6 Increased *C. jejuni* interactions with and invasion of intestinal epithelial cells (IECs) are mirrored by an enhanced host response. After either Caco-2 (A and B) or T84 (C and D) IECs were cocultured with *C. jejuni* 11168H (A and C) or 81-176 (B and D) wild-type strains with either aerobic (Aerobic) or microaerobic (Micro) conditions in the apical compartment, supernatants from apical (Ap) and basolateral (Bl) compartments of a vertical diffusion chamber (VDC) were probed for the presence of the proinflammatory chemokine IL-8 by enzyme-linked immunosorbent assay. Uninfected IECs were used included as controls. ★, $P < 0.05$; ★★, $P < 0.01$.

der microaerobic conditions. Additionally, the *C. jejuni* 11168H wild-type strain was cocultured for 24 h with T84 IECs with either aerobic or microaerobic conditions in the apical compartment (Fig. 5C). After 24 h, there was a significant ($P < 0.05$) 41-fold increase in intracellular *C. jejuni* 11168H bacteria recovered from T84 cells under microaerobic conditions.

Increased *C. jejuni* interactions with IECs during coculture in a VDC result in an increased, polarized innate immune response. The neutrophil chemoattractant interleukin-8 (IL-8) has been shown to be involved in the host innate immune response to *C. jejuni* in both IECs and primary human tissue (26). Caco-2 IECs have been shown to secrete only low levels of IL-8 in response to *C. jejuni* infection, while T84 IECs have been shown to secrete much higher levels of IL-8 (32). Also, the *C. jejuni* 81-176 wild-type strain has been shown to induce a stronger IL-8 response than the 11168H wild-type strain from both Caco-2 and HEp-2 cells (49). IL-8 secretion from Caco-2 or T84 IECs cocultured with *C. jejuni* 11168H or 81-176 in a VDC under either microaerobic or aerobic conditions was assessed. Supernatants from both apical and basolateral VDC compartments were probed separately for the presence of IL-8 (Fig. 6). There was a significant increase in IL-8 secretion from Caco-2 IECs under microaerobic conditions during infection with either 11168H or 81-176, and IL-8 secretion into the basolateral compartment was significantly higher than that into the apical compartment during infection with 81-176 (Fig. 6B). 81-176 induced higher levels of IL-8 secretion from Caco-2 IECs than 11168H. The levels of IL-8 secreted by T84 IECs

in response to either 11168H or 81-176 were much higher than those secreted by Caco-2 IECs under both microaerobic and aerobic conditions (Fig. 6C and D). However, the highest levels of IL-8 secretion were into the basolateral compartment under microaerobic conditions, suggesting that analogous to Caco-2 IECs, T84 IECs respond to increased numbers of interacting *C. jejuni* bacteria by mounting an increased, polarized innate immune response.

Analysis of *C. jejuni* 11168H gene expression after coculture with Caco-2 IECs in the apical compartment of a VDC under either aerobic or microaerobic conditions. In order to investigate bacterial factors involved in the observed increased bacterial interaction and invasion of IECs after coculture in a VDC under microaerobic conditions, gene expression profiles of the *C. jejuni* 11168H wild-type strain in the apical compartment of the VDC after 6 and 24 h of coculture with Caco-2 IECs were analyzed using standard microarray techniques. Based on ANOVA selection methodology, a total of 67 genes were differentially expressed after 6 h under microaerobic conditions compared to aerobic conditions, with 43 genes upregulated and 24 genes downregulated (see Tables S1 and S2 in the supplemental material). Of most significance was the upregulation of *fdhA*, *petA*, and *Cj0414*, suggesting the activation of a different respiratory pathway during coculture under microaerobic conditions. After 24 h, a total of 132 genes were differentially expressed under microaerobic conditions compared to aerobic conditions, with 73 genes upregulated and 59 genes downregulated under microaerobic conditions (see Tables

S3 and S4 in the supplemental material). The *recN*, *mfd*, *rarA*, and *ruvA* genes, encoding DNA repair proteins, were upregulated under aerobic conditions, suggesting greater levels of DNA damage under aerobic conditions. Also, the *Cj1425c*, *Cj1440c*, and *kpsT* genes in the capsular polysaccharide (CPS) locus were downregulated under microaerobic conditions. A recent study demonstrated the downregulation of CPS genes when in contact with IECs *in vitro* (11). Downregulation of CPS genes may lead to greater exposure of *C. jejuni* surface structures that may be involved in mediating bacterial interactions with the IECs. However, the expression of genes encoding many *C. jejuni* virulence factors is unchanged. It is possible that changes in the regulation of bacterial factors involved in the observed increased bacterial interaction and invasion of IECs after coculturing in a VDC under microaerobic conditions occur at the posttranscriptional level and are not reflected in these results.

A nonmotile *C. jejuni* 11168H *rpoN* mutant lacks the ability for enhanced interactions with IECs during coculture in a VDC under microaerobic conditions. Motility has previously been demonstrated to be important for *C. jejuni* interaction and invasion of the intestinal epithelium (36, 44). Despite the lack of significant changes in the expression of flagellar biosynthesis genes observed in the microarray studies, the effect of motility on the observed increased bacterial interaction and invasion of IECs after coculture in a VDC under microaerobic conditions was investigated. A nonmotile 11168H *rpoN* mutant was cocultured with Caco-2 IECs in a VDC with either microaerobic or aerobic conditions in the apical compartment for 6 h. Significantly lower numbers of *rpoN* mutant bacteria than 11168H wild-type bacteria were able to interact with (Fig. 7A) and invade (Fig. 7B) Caco-2 IECs under both microaerobic and aerobic conditions. Most importantly, the *rpoN* mutant had lower levels of interaction with and invasion of Caco-2 IECs under microaerobic conditions than under aerobic conditions, in contrast with the wild-type strain. A complemented 11168H *rpoN* mutant strain was generated by reinsertion of a functional copy of the gene into a predicted pseudogene on the chromosome (25). Successful complementation was demonstrated by restoration of wild-type autoagglutination and motility phenotypes (data not shown). The 11168H *rpoN* complement also partially restored the wild-type phenotype, demonstrating enhanced interaction with and invasion of Caco-2 IECs under microaerobic conditions (Fig. 7A and B).

DISCUSSION

C. jejuni is one of the most prevalent causes of food-borne gastroenteritis worldwide. However, despite the prevalence of this human pathogen, the molecular basis of pathogenicity remains poorly understood in comparison to that for other enteric pathogens. This is partly due to the lack of a convenient, reproducible small-animal model and major drawbacks with the widely used *in vitro* tissue culture cell models (15, 21). To date, *in vitro* tissue culture assays have indicated only very low levels of *C. jejuni* invasion, which does not correlate with the observed clinical symptoms of bloody, inflammatory diarrhea that suggest infection by an invasive enteric pathogen. One of the drawbacks with *in vitro* tissue culture cell assays used to study *C. jejuni* interactions with host cells is the coculturing of the microaerophilic *C. jejuni* with IECs under aerobic conditions, which are likely to result in changes in the ability of the bacteria to interact with IECs. These assay conditions are also not reflective of the very-low-oxygen

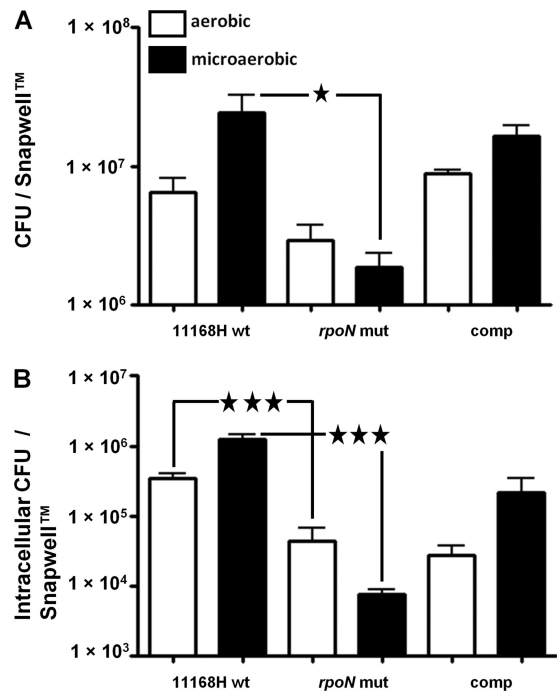


FIG 7 A *C. jejuni* 11168H *rpoN* mutant demonstrates reduced interactions with and invasion of Caco-2 intestinal epithelial cells (IECs) under microaerobic conditions. *C. jejuni* 11168H wild-type (11168H wt), *rpoN* mutant (*rpoN* mut), or complemented *rpoN* (comp) bacteria were cocultured with Caco-2 IECs in a vertical diffusion chamber for 6 h with either aerobic or microaerobic conditions in the apical compartment. The numbers of interacting (A) and intracellular (B) bacteria were assessed. ★, $P < 0.05$; ★★★, $P < 0.001$.

environment in the gut lumen encountered by enteric pathogens during the initial stages of *in vivo* infection of IECs (34). In this study, a modified VDC system was used to allow the coculture of *C. jejuni* with IECs under microaerobic conditions, to provide a more relevant model of the conditions under which *in vivo* infection occurs.

After establishing that both Caco-2 and T84 IECs could be maintained in the VDC with microaerobic conditions in the apical compartment for at least 24 h without any apparent detrimental effects, the effect of coculturing *C. jejuni* 11168H wild-type strain bacteria with IECs under these conditions was assessed. A time-dependent increase in the numbers of both interacting and intracellular *C. jejuni* 11168H wild-type strain bacteria was demonstrated under microaerobic conditions. These results were confirmed using a second *C. jejuni* wild-type strain (81-176) as well as using a second IEC line (T84) to rule out possible strain- or cell line-specific effects. The increased levels of bacterial interaction and invasion were demonstrated to lead to an increased, polarized innate immune response from the IECs. Significantly more IL-8 was detected after coculturing under microaerobic conditions, suggesting that IECs are able to sense and respond to the increased bacterial challenge. In addition, significantly more IL-8 was detected in the basolateral supernatants than in the apical supernatants. This suggests that the IL-8 secretion occurs in a polarized fashion, with the chemokine secreted from the basolateral surface. This concurs with the biological function of IL-8 as a neutrophil attractant, which would be of limited use in the intestinal lumen. In agreement with previous reports (32), a marked

difference in amount of secreted IL-8 was detected between the two IEC lines used, with the T84 IECs demonstrating higher levels of secretion than the Caco-2 IECs. Both *C. jejuni* wild-type strains induced similar levels of IL-8 secretion from T84 IECs but differed in ability to induce an IL-8 response from the Caco-2 IECs, despite demonstrating similar numbers of interacting and invading bacteria. This suggests that both *C. jejuni* strain-specific factors and IEC line specific-factors contribute to the level of the innate immune response observed in these experiments.

A *C. jejuni* NCTC11168 *rpoN* mutant is completely aflagellate, nonmotile, and unable to secrete the CiaB protein (17). In this study, a 11168H *rpoN* mutant exhibited lower numbers of interacting and intracellular bacteria than the wild-type strain when cocultured with Caco-2 IECs in the VDC under either microaerobic or aerobic conditions after 6 h of coculturing. However, lower numbers of intracellular 11168H *rpoN* mutant bacteria were recovered after coculturing under microaerobic conditions. This is in contrast to the *C. jejuni* 11168H wild-type strain, where fewer intracellular bacteria were recovered after coculturing under aerobic conditions. These data suggest that bacterial motility is not just important for the interaction and invasion of *C. jejuni per se* but is also an important factor involved in mediating the increased interaction and invasion of *C. jejuni* when cocultured with Caco-2 IECs in the VDC under microaerobic conditions.

A VDC model system was used to investigate the interactions of the microaerophilic gastric pathogen *H. pylori* with epithelial cells under low-oxygen conditions in the apical compartment, demonstrating increased numbers of adherent bacteria when cocultured with Caco-2 IECs under microaerobic conditions (12). Caco-2 IECs were used as no polarized gastric epithelial cell line was available. A more recent study using a similar VDC model to analyze the interaction of the facultative anaerobe enterohemorrhagic *E. coli* (EHEC) with IECs under anaerobic conditions demonstrated increased interactions of the bacteria when cocultured with anaerobic/microaerobic conditions in the apical compartment of the VDC (42). This suggests that the behavior not only of microaerobic bacteria but also of bacteria that are capable of proliferating at atmospheric oxygen concentration is changed when cocultured with IECs under microaerobic or anaerobic conditions. Using the VDC model with *C. jejuni* allows for the first time the interactions of an invasive enteric bacterial pathogen to be studied under low-oxygen coculture conditions. The data suggest that the VDC model is very useful for analysis of the host-pathogen interactions of a wide range of pathogenic bacteria under conditions more closely resembling the *in vivo* situation in the human intestinal lumen. However, it should be noted that VDC models, like other *in vitro* cell culture models, are limited in the extent to which they model the complexity of real tissue. Further steps need to be taken to more closely represent the complexity of the intestinal epithelium, especially in terms of mucous secretion and different cell types present in the human intestine.

Even though it has been demonstrated that *C. jejuni* can invade and survive within IECs *in vitro*, the fate of the bacteria postinfection has been very difficult to assess due to the small amounts of invasion observed under standard *in vitro* tissue culture conditions (21). Performing such coculture experiments in the VDC model system increased by 80-fold the number of intracellular bacteria observed after 24 h. This allows for a much more detailed analysis of mechanisms of IEC invasion by *C. jejuni*, as invasion is no longer a rare event. Furthermore, the intracellular fate of *C.*

jejuni is more easily traceable. Methods, such as analysis of gene transcription from intracellular bacteria, that have not been possible to date due to the amounts of recoverable RNA being below a useful threshold should now be possible. Furthermore, as the compartment in which the bacteria are incubated is separate from the one supporting the IECs with nutrients and oxygen, it is more amenable to manipulations than classical coculturing in tissue culture plates without interfering with the IECs. This means that it will be easier to test the effects of different substances on the invasive behavior of the bacteria. Substances, like bile salts, that have been shown to increase expression of *C. jejuni* virulence genes can be added to the bacterial suspension and their effect on bacterial invasion, host response, or monolayer disruption analyzed.

Using the VDC model system to coculture *C. jejuni* with IECs under microaerobic conditions resulted in dramatic changes in the host-pathogen interactions observed. This model provides an improved mimic of the *in vivo* situation encountered by *C. jejuni* in the human intestinal lumen. IECs are not negatively affected by microaerobic conditions at the apical surface over 24 h. A time-dependent increase in the numbers of both interacting and intracellular *C. jejuni* bacteria was demonstrated after coculturing with Caco-2 IECs in the VDC under microaerobic conditions. This increased interaction of *C. jejuni* with the IECs was mirrored by an increased innate immune response. Taken together, these results indicate that use of the VDC model system provides an improved model to investigate *C. jejuni*-host cell interactions and the elucidation of the molecular basis of pathogenesis.

ACKNOWLEDGMENTS

Dominic C. Mills was supported by a Bloomsbury Colleges Ph.D. studentship (2007 to 2010) awarded to N.D. and P.W.T.

We thank Dennis Linton (University of Manchester, United Kingdom) for providing the *C. jejuni* NCTC11168 complementation vector. We acknowledge BμG@S (the Bacterial Microarray Group at St. George's, University of London) for supply of the microarray and advice and The Wellcome Trust for funding this multicollaborative microbial pathogen microarray facility under the Functional Genomics Resources Initiative.

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