Increase in *Campylobacter jejuni* invasion of intestinal epithelial cells under low oxygen co-culture conditions that reflect the *in vivo* environment

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Running title: Increase in *Campylobacter jejuni* invasion

Abstract

Campylobacter jejuni infection often results in bloody, inflammatory diarrhea, indicating bacterial disruption and invasion of the intestinal epithelium. Whilst *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 is neither 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 baso-lateral surface of cultured IECs producing an *in vitro* system that closely mimics *in vivo* conditions in the human intestine. Nine-fold increases in interacting and eighty-fold increases in intracellular *C. jejuni* 11168H wild-type strain bacteria were observed after 24 hours co-culture with Caco-2 IECs in VDCs with microaerobic conditions at the apical surface compared to aerobic conditions. Increased bacterial interaction was matched by an enhanced and directional host innate immune response, particularly an increased baso-lateral secretion of the pro-inflammatory chemokine IL-8. Analysis of the invasive ability of a non-motile *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.

Introduction

The Gram-negative, microaerophilic *Campylobacter jejuni* is one of the most common causes of foodborne bacterial gastroenteritis in developed countries (6), with 500,000 and 2,500,000 predicted cases each year in the UK and USA respectively (27). The predominant route of transmission is by 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 post-infectious sequelae such as Guillain-Barré Syndrome, reactive arthritis and Inflammatory Bowel Disease (IBD) (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 when compared to 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, as well as 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 successfully used in various studies (18, 24), such studies reflect *C. jejuni* colonisation and the direct relevance of the chick and chicken models relating to human Campylobacteriosis 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 pre-treatments of the mice, the routes of inoculation and the inoculation loads have resulted in findings as diverse as non-colonization, non-symptomatic carriage or severe diarrhea (3, 8, 43, 48). Knock-out mice models of *C. jejuni* enteritis using mice deficient in NF-κB (19), MyD88 (45), interleukin-10 (33) and Nramp1 (9) have been reported. Additionally, infections of mice with limited enteric flora have also been reported (10). The outcome of *C. jejuni* infection in these models differs 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 polarised and non-polarised intestinal epithelial cells (IECs) *in vitro* including 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 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 co-culturing 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 defence mechanisms against oxidative stress such as the SodB superoxide dismutase (39) and the KatA catalase (14), it is likely that the bacterium will behave differently under atmospheric oxygen conditions than in the natural low oxygen environment of the intestinal lumen.

When the microaerophilic human pathogen *Helicobacter pylori* was co-cultured with epithelial cells with microaerobic conditions at the apical surface and aerobic conditions at the baso-lateral surface using a Vertical Diffusion Chamber (VDC) or Ussing chamber, the result was a significant increase in bacterial adherence under microaerobic conditions compared to 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 more closely mimic the *in vivo* situation and as such should allow more accurate investigations of host-pathogen interactions. In this study, a modified VDC system was used to allow the co-culture of *C. jejuni* with IECs under microaerobic conditions in the apical compartment, resulting in an eighty-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 colonisation levels in a chick colonisation model than the NCTC11168 strain (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, UK) and 7% (v/v) horse blood (TCS Microbiology, Botolph Claydon, UK) at 37°C in a VAIN (Variable Atmosphere INcubator) microaerobic chamber (Don Whitley Scientific, Sheffield, UK) containing 85% N_2, 10% CO_2 and 5% O_2. Appropriate antibiotics were added at the following concentrations; 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 (UK) unless otherwise stated.

Epithelial cell line and culture conditions

The human Caco-2 IEC line was cultured in Dulbecco's modified essential media (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS; Sigma-Aldrich, Poole, UK), 100 U/ml penicillin, 100 μg/ml streptomycin and 1% (v/v) non-essential amino acids and maintained at 37°C in 5% CO_2 and 95% air. The human T84 IEC line was cultured in 1:1 DMEM/F12 medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) FCS and 100 U/ml penicillin, 100 μg/ml streptomycin. For VDC experiments, 4 x 10^5 Caco-2 or T84 IECs were

seeded into the upper compartment of a Snapwell™ filter (Corning Lifesciences, Amsterdam, The Netherlands). To allow for the formation of a polarised monolayer, cells were grown for a minimum of 21 days for Caco-2 IECs and 14 days for T84 IECs respectively. 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, USA) were sterilised by immersion in Haz-Tabs (Guest Medical Ltd, Aylesford, UK) solution for 3 h, followed by a three washes with sterile water. A Snapwell™ filter carrying a polarised 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 baso-lateral compartment was filled with 4 ml of the appropriate tissue culture medium supplemented with 1% (v/v) FCS and 1% (v/v) non-essential amino acids. The apical compartment was filled with 4 ml Brucella broth (Oxoid). For infections, approximately 1 x 10^9 *C. jejuni* were harvested from a 24 h BA plate and added to the apical compartment. For aerobic co-culturing, the VDC was maintained at 37°C in 5% CO_2 and 95% air. For microaerobic co-culturing, the VDC was maintained under microaerobic conditions (85% N_2, 10% CO_2 and 5% O_2) in a VAIN. A gas mixture of 95% O_2 and 5% CO_2 was perfused through the baso-lateral compartment, while the apical compartment was left open to the microaerobic atmosphere in the VAIN (Figure 1).

Immunofluorescence analysis of cellular distribution of actin and occludin

IECs were fixed with 2% (w/v) paraformaldehyde (Sigma-Aldrich) for 1 h at 4°C, permeabilised with 0.1% (v/v) Triton X-100 in PBS for 20 min then blocked with 1% (w/v) 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 1:1000 in PBS) for 1 h in the dark. For occluding staining, IECs were incubated with mouse anti-occludin primary antibody (stock diluted 1:100 in PBS) (Invitrogen) for 1 h at room temperature followed by an 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 µg/ml) (Vector Laboratories, Peterborough, UK) on a coverslip (Fisher Scientific, Loughborough, UK) 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, placed back into the hanging support and 500 µl of 100 µM FITC-labelled dextran (Sigma-Aldrich) with an average molecular weight of 4 kilodaltons (kDa) in Ringer's solution (115 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$) added to the apical side of the monolayer and incubated for 3 h at room temperature, with the baso-lateral side of the monolayer immersed in Ringer's solution. The amount of fluorescently labelled dextran on the baso-lateral side of the monolayer was determined post-incubation by removal of the baso-lateral solution and measurement of the fluorescence intensity at 488 nm using a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, USA).

Measurement of Caco-2 monolayer Trans-Epithelial Electrical Resistance in the VDC

The trans-epithelial 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) 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 co-culturing, the apical and baso-lateral 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% (v/v) Triton X-100 in PBS for 20 minutes 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 calculated. Enumeration of intracellular bacteria was essentially performed as above, with the following modification. Before lysis with Triton X-100, the IECs were incubated in DMEM containing 150 μg/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 co-culturing. 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 co-culturing supernatants

Co-culturing supernatants were probed for the presence of Interleukin 8 (IL-8) with the Human IL-8 ELISA Development Kit (Peprotech, London, UK), 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 co-culturing with Caco-2 IECs for 6 h or 24 h under either aerobic or microaerobic conditions were analysed using an indirect comparison method or type 2 experimental design (46). Replicate test sets of Cy5-labelled *C. jejuni* 11168H total RNA samples were combined with a common reference sample (Cy3-labelled *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 BμG@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, USA). Quantified data were analysed using GeneSpring GX software v7.3 (Agilent, Santa Clara, USA). Statistically significant up- and down-regulated genes were selected when comparing 11168H gene expression under microaerobic conditions against aerobic conditions using ANOVA (ANalysis Of VAriance) 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 using a *C. jejuni* NCTC11168 complementation vector (25) using previously described techniques (23). The complementation vector utilises 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 proof-reading *Pfu* polymerase (Fermentas, Sankt Leon-Rot, Germany) and ligated into the *Nco*I and *Nhe*I 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 was statistically analysed using Prism software (GraphPad Software Inc., La Jolla, USA). Figures display means ± standard error as well as the *p* – values of 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 BμG@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 has been deposited in BμG@Sbase (accession number E-BUGS-125; http://bugs.sgul.ac.uk/E-BUGS-125) and also ArrayExpress (accession number 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 co-culture *C. jejuni* and IECs with microaerobic conditions at the apical surface, the effect of these conditions on polarised IECs was determined. The morphology of Caco-2 monolayers after 24 h incubation in a VDC was analysed by confocal laser microscopy. No difference was detected in the distribution of actin between IECs that had been maintained under microaerobic conditions (Figure 2B) compared to under aerobic conditions (Figure 2A). The fluorescence signal localised predominantly to the junctions between adjacent cells. Additionally, a strong localisation of actin to the apical side of the Caco-2 IECs was noted, indicating the formation of a dense microvilli brush border. This indicated 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 analysed. No difference was detected in the distribution of occludin between IECs that had been maintained under microaerobic conditions (Figure 2B) compared to under aerobic conditions (Figure 2A). Fluorescence was tightly localised to the cell-cell boundaries, indicating the presence of intact tight junctions. A similar distribution in actin and occludin was observed with T84 IECs (data not shown).

The barrier function of polarised 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 VDC, the diffusion of fluorescently labelled dextran across Caco-2 (Figure 3A) and T84 (Figure 3B) monolayers was determined. Approximately 25% of the labelled dextran diffused across an empty

Snapwell™ filter after 3 h. However there was no significant difference in dextran diffusion between IECs grown on Snapwell™ filter 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 permeabilisation with Triton X-100, demonstrating that it is the barrier function of the polarised IECs that prevents dextran diffusion. Measuring the trans-epithelial electrical resistance (TEER) of an IEC monolayer on a permeable support is a direct, quantitative method for analysis of the polarisation 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 aerobic conditions (Figure 3C).

C. jejuni interactions with IECs are enhanced during co-culture in a VDC under microaerobic conditions

C. jejuni 11168H wild-type strain was co-cultured with Caco-2 IECs at an MOI of approximately 100:1 with either microaerobic or aerobic conditions in the apical compartment for 3, 6 and 24 h (Figure 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 was observed under microaerobic conditions compared to 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 co-culturing (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 that then become more susceptible to *C. jejuni* infection.

C. jejuni invasion of IECs is dramatically enhanced during co-culture in a VDC under microaerobic conditions

C. jejuni 11168H wild-type strain was co-cultured 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 determined (Figure 5A). There was a significant ($p < 0.05$) 5-fold increase after 6 h in the numbers of intracellular *C. jejuni* under microaerobic conditions. After 24 h, there was a significant ($p < 0.01$) 80–fold increase in intracellular *C. jejuni* recovered under microaerobic conditions. To confirm that the observed increase in numbers of intracellular *C. jejuni* after 24 h of co-culturing 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 co-cultured for 24 h with Caco-2 IECs with either aerobic or microaerobic conditions in the apical compartment (Figure 5B). After 24 h, there was a significant ($p < 0.05$) 89–fold increase in intracellular *C. jejuni* 81-176 recovered from Caco-2 cells under microaerobic conditions. Additionally, *C. jejuni* 11168H wild-type strain was co-cultured for 24 h with T84 IECs with either aerobic or microaerobic conditions in the apical compartment (Figure 5C). After 24 h, there was a significant ($p < 0.05$) 41–fold increase in intracellular *C. jejuni* 11168H recovered from T84 cells under microaerobic conditions.

Increased _C. jejuni_ interactions with IECs during co-culture in a VDC results in an increased, polarised 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, whilst T84 IECs have been shown to secrete much higher levels of IL-8 (32). Also *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 co-cultured with *C. jejuni* 11168H or 81-176 in a VDC under either microaerobic or aerobic conditions was assessed. Supernatants from both apical and baso-lateral VDC compartments were probed separately for the presence of IL-8 (Figure 6). There was a significant increase in IL-8 secretion from Caco-2 IECs under microaerobic conditions when infected with either 11168H or 81-176 and IL-8 secretion was significantly higher into the baso-lateral compartment than into the apical compartment during infection with 81-176 (Figure 6B). 81-176 induced higher levels of IL-8 secretion from Caco-2 IECs than 11168H. Compared to Caco-2 IECs, the levels of IL-8 secreted by T84 IECs in response to either 11168H or 81-176 were much higher under both microaerobic and aerobic conditions (Figure 6CD). However the highest levels of IL-8 secretion were into the baso-lateral compartment under microaerobic conditions, suggesting that analogous to Caco-2 IECs, T84 IECs respond to increased numbers of interacting *C. jejuni* by mounting an increased, polarised innate immune response.

Analysis of *C. jejuni* 11168H gene expression after co-culturing 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 co-culturing in a VDC under microaerobic conditions, gene expression profiles of *C. jejuni* 11168H wild-type strain in the apical compartment of

the VDC after 6 and 24 h of co-culturing with Caco-2 IECs were analysed using standard microarray techniques. Based on an 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 (Supplementary Tables S1-2). Of most significance was the up-regulation of *fdhA*, *petA* and *Cj0414*, suggesting the activation of a different respiratory pathway during co-culturing 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 (Supplementary Tables S3-4). The *recN*, *mfd*, *rarA* and *ruvA* genes encoding DNA repair proteins were up-regulated 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 down-regulated under microaerobic conditions. A recent study demonstrated the down-regulation of CPS genes when in contact with IECs *in vitro* (11). Down-regulation of CPS 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 are unchanged. It is possible that changes in the regulation of bacterial factors involved in the observed increased bacterial interaction and invasion of IECs after co-culturing in a VDC under microaerobic conditions occur at the post-transcriptional level and would not be reflected in these results.

A non-motile *C. jejuni* 11168H *rpoN* mutant lacks the ability for enhanced interactions with IECs during co-culture 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 co-culturing in a VDC under microaerobic conditions was investigated. A non-motile 11168H *rpoN* mutant was co-cultured 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 were able to interact with (Figure 7A) and invade (Figure 7B) Caco-2 IECs compared to the 11168H wild-type strain 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 re-insertion 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 (Figures 7A and 7B).

Discussion

C. jejuni is one of the most prevalent causes of foodborne gastroenteritis worldwide. However, despite the prevalence of this human pathogen, the molecular basis of pathogenicity remains poorly understood in comparison to 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 co-culturing 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 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 co-culture 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 co-culturing *C. jejuni* 11168H wild-type strain bacteria with IECs under these conditions was assessed. A time-dependent increase of 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, polarised innate immune response from the IECs. Significantly more IL-8 was detected after co-culturing 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 baso-lateral supernatants compared to the apical supernatants. This suggests that the IL-8 secretion occurs in a polarised fashion, with the chemokine secreted from the baso-lateral 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 the amount of secreted IL-8 was detected between the two IEC lines used, with the T84 IECs demonstrating higher levels of secretion compared to the Caco-2 IECs. Both *C. jejuni* wild-type strains induced similar levels of IL-8 secretion from T84 IECs, but differed in the 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, non-motile and unable to secrete the CiaB protein (17). In this study a 11168H *rpoN* mutant exhibited lower numbers of interacting and intracellular bacteria compared to the wild-type strain when co-cultured with Caco-2 IECs in the VDC under either microaerobic or aerobic conditions after 6 h of co-culturing. However lower numbers of intracellular 11168H *rpoN* mutant bacteria were recovered after co-culturing under microaerobic conditions. This is in contrast to the *C. jejuni* 11168H wild-type strain, where less intracellular bacteria were recovered after co-culturing under aerobic conditions. This data suggests 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 co-cultured 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 co-cultured with Caco-2 IECs under microaerobic conditions (12). Caco-2 IECs were used as no polarised gastric epithelial cell line was available. A more recent study using a similar VDC model to analyse the interaction of the facultative anaerobe EHEC with IECs under anaerobic conditions demonstrated increased interactions of the bacteria when co-cultured with anaerobic/microaerobic conditions in the apical compartment of the VDC (42). This suggests that the behaviour not only of microaerobic bacteria but also of bacteria that are capable of proliferating at atmospheric oxygen concentration is changed when co-cultured 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 co-culture conditions. The data suggests that the VDC model is a very useful model 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 post-infection has been very difficult to assess due to the low amounts of invasion observed under standard *in vitro* tissue culture conditions (21). Performing such co-culture experiments in the VDC model system increased by 80-fold the

number of intracellular bacteria observed after 24 h. This will allow 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* will be 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 co-culturing in tissue culture plates without interfering with the IECs. This means that it will be easier to test the effect of different substances on the invasive behaviour 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 analysed.

Using the VDC model system to co-culture *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 of the numbers of both interacting and intracellular *C. jejuni* was demonstrated after co-culturing 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.

Acknowledgements

Dominic Mills was supported by a Bloomsbury Colleges PhD Studentship (2007-2010) awarded to ND and PWT. We thank Dennis Linton (University of Manchester, UK) 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 multi-collaborative microbial pathogen microarray facility under the Functional Genomics Resources Initiative.

Figure Legends

Figure 1. The Vertical Diffusion Chamber (VDC) model system. IECs (IECs) were grown to polarisation on Snapwell™ filters and placed between the two compartments of the VDC. The apical compartment was filled with Brucella broth and the baso-lateral compartment was filled with tissue culture media. For infection studies, *C. jejuni* were 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% CO_2. For microaerobic co-culturing, the VDC was placed into a microaerobic incubator with the apical compartment open to the atmosphere within the incubator and the baso-lateral compartment closed and perfused with 95% O_2 and 5% CO_2.

Figure 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 hours. 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), actin (red) and the nuclei counterstained with DAPI (blue). The images represent projections of a stack of Z-axis slices viewed from above.

Figure 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 (3A and 3C) or T84 (3B) 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 and 500 µl of 100 µM FITC-labelled dextran (average molecular weight of 4 kilodaltons) in Ringer's solution was added to the apical side of the monolayer and incubated for 3 hours at room temperature (3A and 3B). After 3 hours, the percentage of FITC-labelled dextran that had passed across the monolayer was determined from the relative fluorescence of the baso-lateral solution and the relative fluorescence of the input solution. An empty Snapwell™ filter (No cells), IECs grown for 21 days on Snapwell™ filters and permeabilised with 0.5% (v/v) Triton X-100 for 20 mins at room temperature (Triton) and IECs grown for 21 days on Snapwell™ filters (Un) were used as controls. The trans-epithelial electrical resistance (TEER) was measured after assembly of the VDCs and set as 100% (3C). After 3h, 6h and 24 h post-assembly, the TEER was measured and calculated as a percentage of the value obtained at time point 0.

Figure 4. *C. jejuni* interactions with intestinal epithelial cells (IECs) are significantly increased under microaerobic conditions. *C. jejuni* 11168H wild-type strain bacteria were co-cultured 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. * = $p < 0.05$.

Figure 5. *C. jejuni* invasion of intestinal epithelial cells (IECs) is significantly increased under microaerobic conditions. *C. jejuni* wild-type strains were co-cultured 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) 11168 co-cultured with Caco-2 IECs for 6 h and 24 h. (B) 11168H or 81-176 co-cultured with Caco-2 IECs for 24 h. (C) 11168H co-cultured with Caco-2 or T84 IECs for 24 h. * = $p < 0.05$, ** = $p < 0.01$.

Figure 6. Increased *C. jejuni* interactions with and invasion of intestinal epithelial cells (IECs) is mirrored by an enhanced host response. Supernatants from apical (Ap) and baso-lateral (Bl) compartments of a Vertical Diffusion Chamber (VDC) after either Caco-2 (A & B) or T84 (C & D) IECs were co-cultured with *C. jejuni* 11168H (A & C) or 81-176 (B & D) wild-type strains with either aerobic (Aerobic) or microaerobic (Micro) conditions in the apical compartment were probed for the presence of the pro-inflammatory chemokine IL-8 by enzyme-linked immune-sorbent assay. Uninfected IECs were used included as controls. * = $p < 0.05$, ** = $p < 0.01$.

Figure 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, *rpoN* mutant or complemented *rpoN* bacteria were co-cultured 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. * = $p < 0.05$, ** = $p < 0.001$.

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Fig 1.

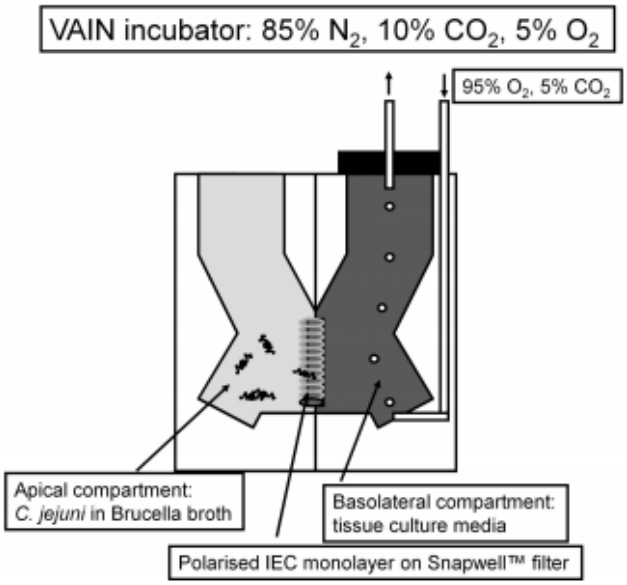
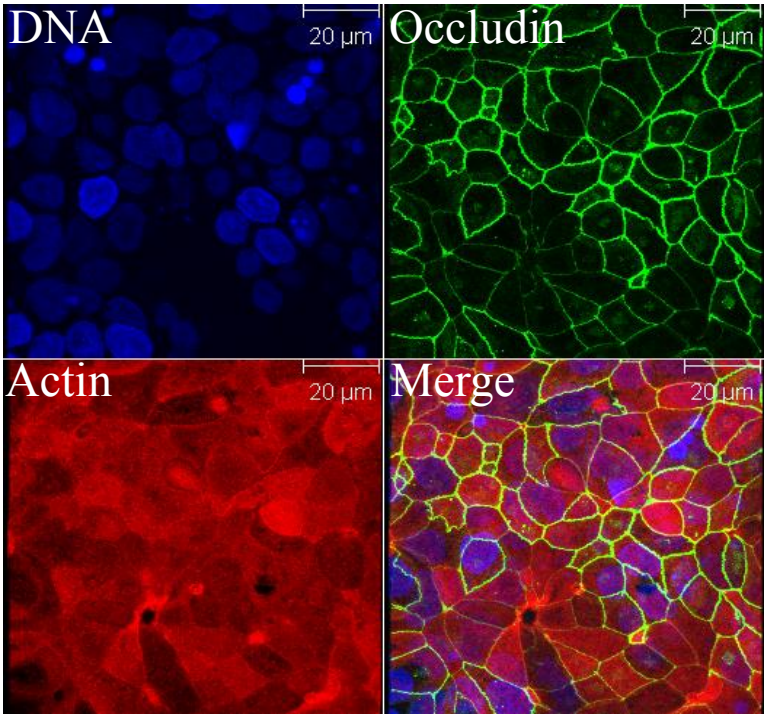

VAIN incubator: 85% N_2, 10% CO_2, 5% O_2
95% O_2, 5% CO_2
Apical compartment:
C. jejuni in Brucella broth
Basolateral compartment:
tissue culture media
Polarised IEC monolayer on Snapwell™ filter

Fig 2A.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Merge
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Fig 2B.

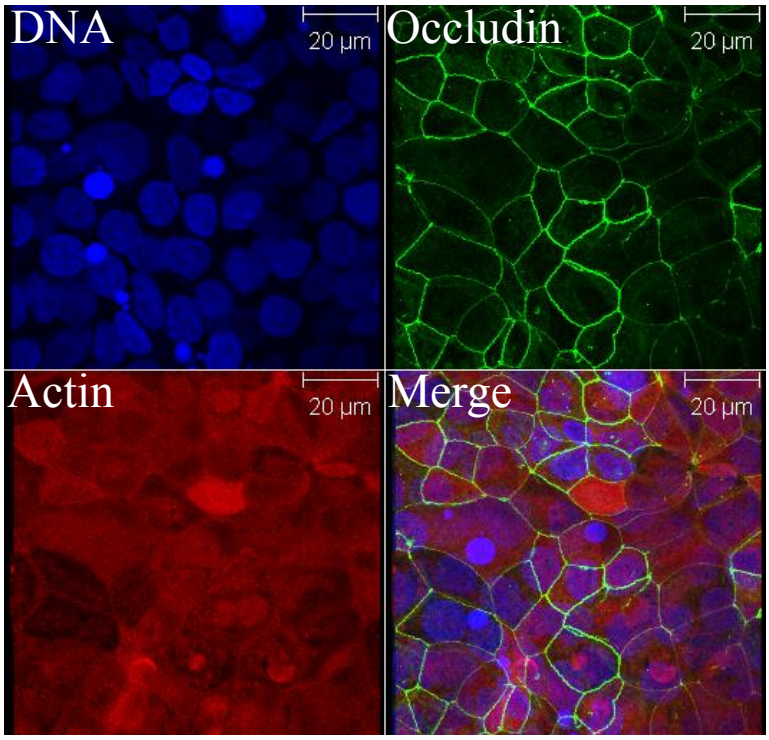
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Fig 3A

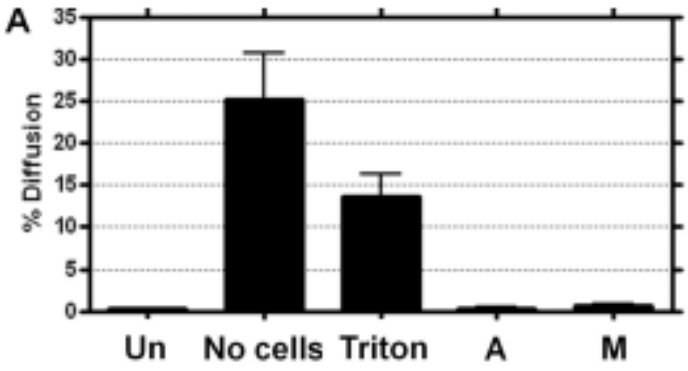
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Fig 3B

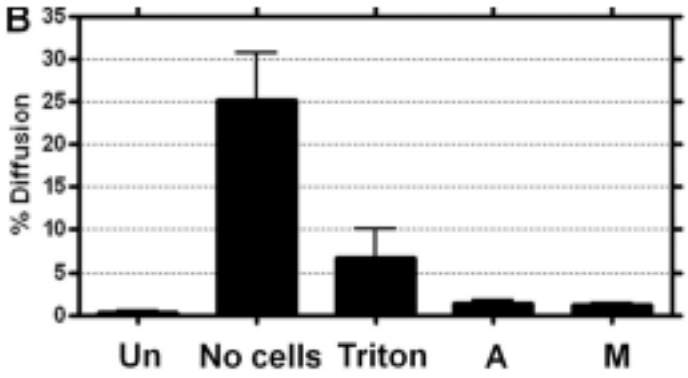
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Fig 3C

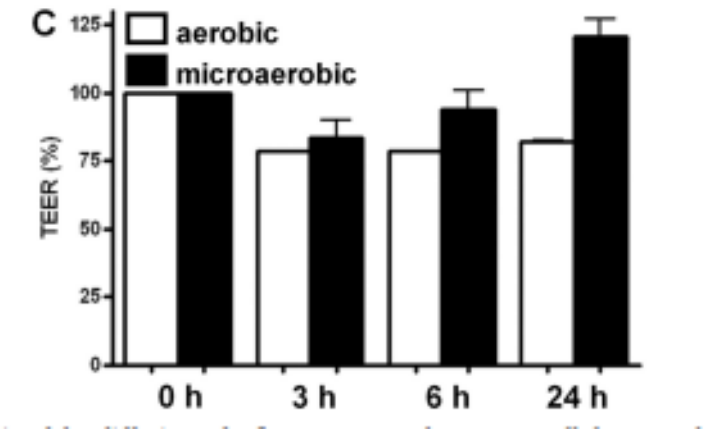
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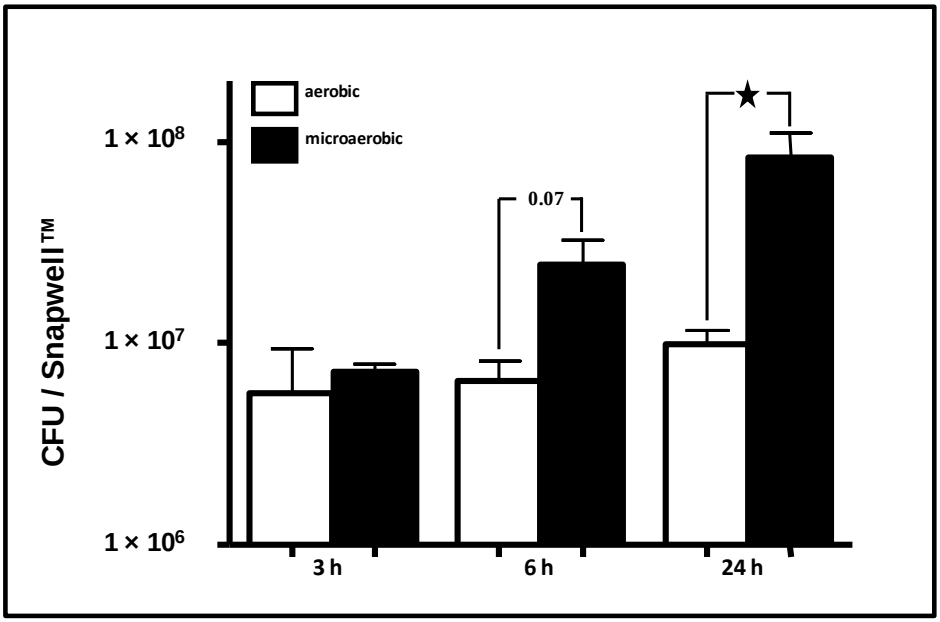
Fig 4.



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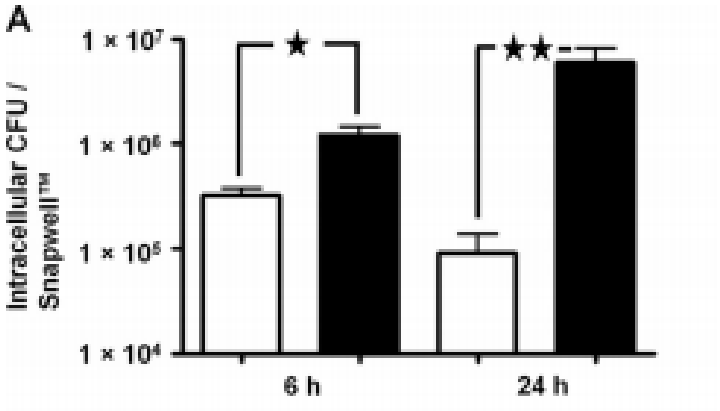

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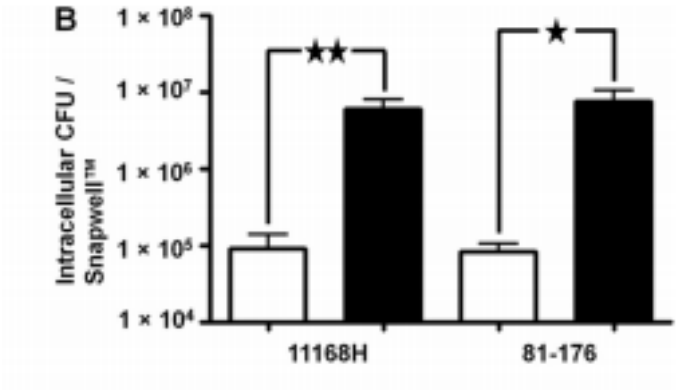
B
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Intracellular CFU / Snapwell™
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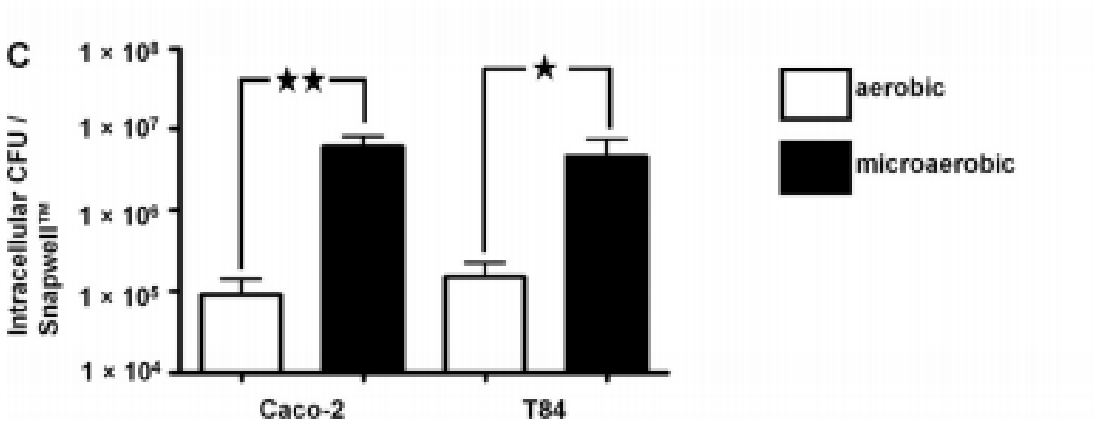
11168H
81-176

Fig 5C.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Intracellular CFU / Snapwell™
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aerobic
microaerobic

Fig 6

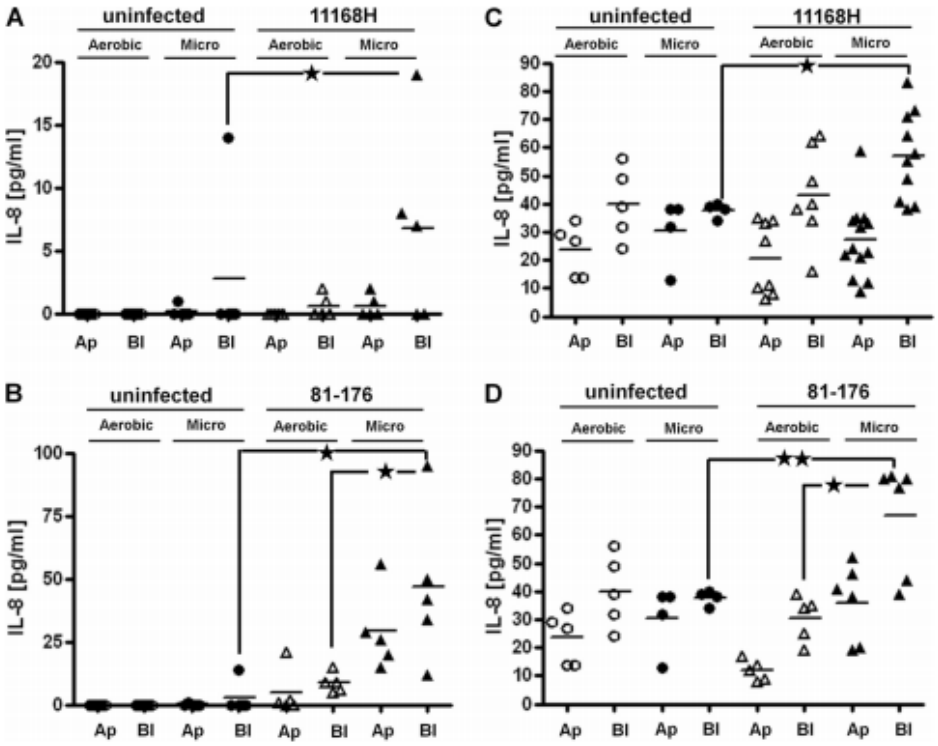

A
uninfected
11168H
Aerobic
Micro
Aerobic
Micro
IL-8 [pg/ml]
Ap
Bl
B
uninfected
81-176
C
uninfected
11168H
D
uninfected
81-176

Fig 7A.

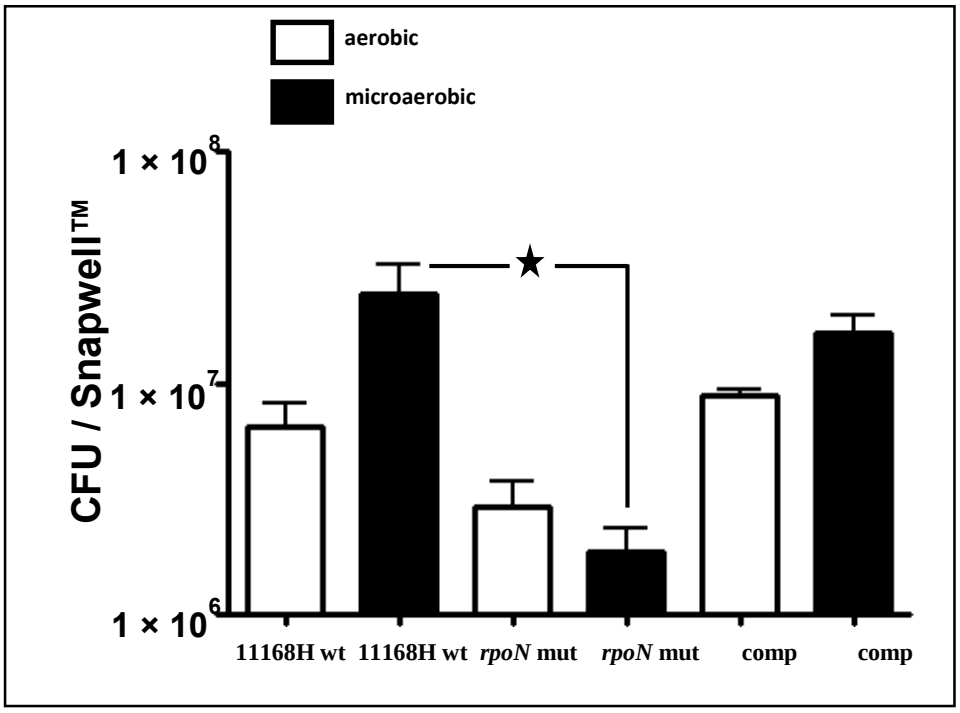
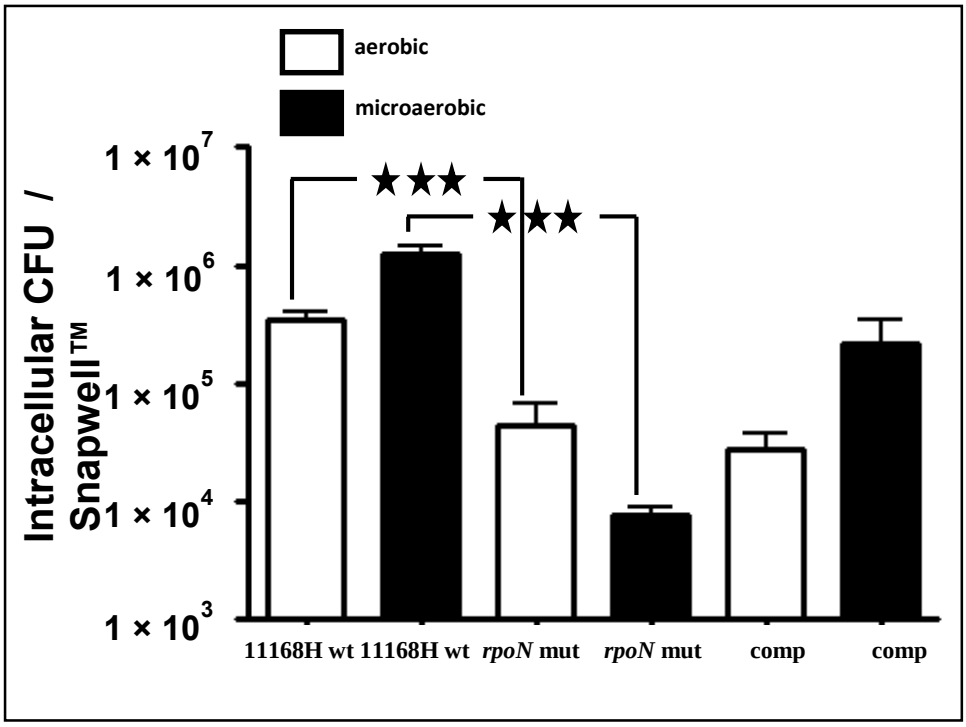

aerobic
microaerobic
CFU / Snapwell™
1 × 10^8
1 × 10^7
1 × 10^6
11168H wt
11168H wt
rpoN mut
rpoN mut
comp
comp

Fig 7B.


aerobic
microaerobic
Intracellular CFU / Snapwell™
1 × 10^7
1 × 10^6
1 × 10^5
1 × 10^4
1 × 10^3
★★★
★★★
11168H wt
11168H wt
rpoN mut
rpoN mut
comp
comp