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Microaerobic conditions enhance type III secretion and adherence of enterohaemorrhagic *Escherichia coli* to polarized human intestinal epithelial cells

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Summary

Advances in the understanding of the pathogenesis of enterohaemorrhagic *Escherichia coli* (EHEC) have greatly benefited from the use of human epithelial cell lines under aerobic conditions. However, in the target site of EHEC infection, the human intestine, conditions are microaerobic. In our study we used polarized human colon carcinoma cells in a vertical diffusion chamber system to investigate the influence of reduced apical oxygen levels on EHEC colonization. While apical microaerobiosis did not affect cell integrity and barrier function, numbers of adherent bacteria were significantly increased under low compared with high apical oxygen concentrations. In addition, expression and translocation of EHEC type III secreted (T3S) effector proteins was considerably enhanced under microaerobic conditions and dependent on the presence of host cells. Increased colonization was mainly mediated via EspA as adherence levels of an isogenic deletion mutant were not influenced by low oxygen levels. Other potential adherence factors (*E. coli* common pilus and flagella) were only minimally expressed under high and low oxygen levels. Addition of nitrate and trimethylamine *N*-oxide as terminal electron acceptors for anaerobic respiration failed to further increase bacterial colonization or T3S under microaerobiosis. This study indicates that EHEC T3S and colonization are enhanced by the microaerobic environment in the gut and therefore might be underestimated in conventional aerobic cell culture systems.

Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) is a human intestinal pathogen that causes diarrhoea and can induce severe complications such as haemorrhagic colitis and haemolytic uraemic syndrome associated with the production of Shiga toxin (Tarr *et al.*, 2005). After ingestion via contaminated food or water, EHEC colonizes the intestinal mucosa by forming attaching/effacing lesions (A/E), which are characterized by intimate bacterial adherence and localized microvillous destruction (Knutton *et al.*, 1987). The A/E lesion formation has been attributed to the presence of a type III secretion (T3S) system that is encoded on the locus of enterocyte effacement (LEE) pathogenicity island and enables the bacterium to inject effector proteins into the host cell (reviewed by Frankel *et al.*, 1998). After establishment of initial contact via EspA containing filaments, two further effector proteins,

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EspB and D, are translocated into the host cell membrane where they form a pore structure (Ebel *et al.*, 1998; Knutton *et al.*, 1998; Ide *et al.*, 2001). Delivery of the translocated intimin receptor (Tir) into the host cell membrane is followed by dissolution of the EspA filament and intimate bacterial attachment via binding of Tir to the bacterial adhesin intimin (Kenny *et al.*, 1997a; Deibel *et al.*, 1998). *In vitro* cell culture models have been useful in elucidating the mechanisms of A/E lesion formation; however, they generally do not consider the altered environment that EHEC encounters *in vivo*. Apart from high osmolarity, 37°C temperature, neutral to alkaline pH and different concentrations of ions and nutrients than in the ecological environment, the milieu in the intestinal tract is characterized by low oxygen levels (He *et al.*, 1999). Studies on enteropathogenic *Salmonella* ssp. have shown that anaerobic conditions increase bacterial adherence, invasion and virulence (Ernst *et al.* 1990; Lee and Falkow, 1990; Schiemann and Shope, 1991; Singh *et al.*, 2000). Whereas most of these studies have used anaerobic bacterial cultures to infect cells in an aerobic environment, we have employed a system that allows apical infection under microaerobic conditions (oxygen concentrations of 1–2% atmospheric pressure) while basolateral cell compartments are maintained under oxygenated conditions. Using this system, we show increased EHEC adherence and T3S under apical microaerobic conditions.

Results

Establishment of a vertical diffusion chamber system for microaerobic EHEC infection

A vertical diffusion chamber system was used to generate an environment that mimics the microaerobic milieu at the luminal side of the intestinal mucosa. Polarized T84 cells were mounted between half chambers resulting in separation of apical and basolateral sides of the monolayer. Apical chambers were perfused with either anaerobic or oxygenated gas mixtures whereas basolateral compartments were maintained under oxygenated conditions to ensure optimal cell survival (Fig. 1). Cells were infected apically with EHEC for 6 h and apical media were exchanged every 2 h to maintain bacteria in early to midlogarithmic growth phase.

Oxygen concentrations in apical chambers were monitored during the experiment and were found to be 90–92% (percentage of atmospheric pressure) under oxygenated and 1–1.7% under apical anaerobic perfusion, thus generating a microaerobic environment. Oxygen concentrations of standing bacterial overnight cultures used for infection were close to 0% at the bottom of the tube and around 4% at the top. Determination of transepithelial electrical resistance (TER) of cell monolayers before and after the experiment showed that neither microaerobic apical conditions nor EHEC infection affected epithelial barrier function and monolayer integrity as TER remained stable under all conditions (data not shown). This was corroborated by occludin staining that showed intact tight junctions after 6 h of infection under microaerobic or oxygenated conditions (Fig. 2A). Scanning electron microscopy (SEM) of membrane filters showed a distinctive brush border of short microvilli and loose bacterial microcolonies on infected cells (Fig. 2B). This was similar under high and low oxygen levels.

Apical microaerobiosis enhances EHEC colonization

Bacterial adherence to polarized T84 monolayers was quantified by plating out serial dilutions of cell lysates and determining colony-forming units (cfu). As shown in Fig. 3A, numbers of adherent EHEC were significantly increased under apical microaerobic conditions compared with an oxygenated environment ($P = 0.0004$). This effect was specific as it was not observed with the nonpathogenic *E. coli* strain HB101 that adhered poorly independent of oxygen levels applied [483.5 ± 47.4 cfu/filter oxygenated (OX) versus 489.8 ± 40.1 cfu/filter microaerobic (MA); mean $\pm$ SEM, $n = 4$, $P > 0.05$]. Despite increased adherence under microaerobiosis, growth of non-adherent EHEC in the apical medium (as assessed by OD₆₀₀) was slower under microaerobic than under oxygenated conditions (Fig. 3B, $P = 0.0001$).

EHEC T3S protein expression and secretion are increased under microaerobic conditions

Previous studies have shown that the T3S effector protein EspA is involved in initial contact and essential for EHEC adherence to epithelial cells (Ebel *et al.*, 1998). Therefore, we investigated whether increased bacterial adherence under microaerobic conditions was associated with enhanced T3S. Media from apical diffusion chambers were sampled after 6 h of infection and supernatant proteins were analysed by SDS-PAGE and Western blotting. Staining of polyacrylamide gels showed that four protein bands were induced under microaerobiosis whereas two bands were evident under oxygenated conditions only (Fig. 4A). Western blotting revealed that three out of the four proteins induced by low oxygen levels were Tir, EspA and EspB (Fig. 4B). Analysis of lysates from non-adherent bacteria in apical media revealed that increased amounts of T3S proteins in supernatants were associated with enhanced protein expression in the bacterial cell (Fig. 4C). We also examined T3S protein expression by adherent EHEC by analysing cell lysates of infected T84 monolayers and found that EspA expression was enhanced under microaerobic conditions (Fig. 4C). This was confirmed by immunofluorescence staining that showed pronounced EspA filament formation on adherent EHEC under microaerobic conditions whereas fewer and shorter filaments were observed under oxygenated conditions (Fig. 4D). In addition to EspA, we also examined the influence of oxygen levels on expression of other potential EHEC adherence factors, i.e. *E. coli* common pilus (ECP) and flagella. Bacterial and cell lysates were analysed by SDS-PAGE and Western blotting, but no specific signal was observed under either oxygenated or microaerobic conditions (data not shown). Immunofluorescence staining of infected T84 monolayers showed that only few adherent bacteria expressed ECP ($12.5 \pm 1.9\%$ OX versus $11.9 \pm 2.3\%$ MA; mean $\pm$ SEM, $n = 4$, $P > 0.05$) or flagella ($1.8 \pm 0.2\%$ OX versus $1.5 \pm 0.3\%$ MA; mean $\pm$ SEM, $n = 4$, $P > 0.05$) independent of oxygen levels employed (Fig. 4D).

Microaerobiosis promotes Tir translocation into the host cell

Having demonstrated increased EspA filament formation under microaerobic conditions we wanted to determine whether this was associated with increased Tir translocation into the host cell. Immunofluorescence staining of infected T84 cells demonstrated that very few bacteria showed Tir translocation after 6 h of infection (data not shown). For this reason we performed infections of polarized Caco-2 cells where EHEC A/E lesion formation was

observed at higher frequency. As shown for T84 cells, Caco-2 cell viability and barrier function was unaffected by apical microaerobiosis or EHEC infection for 6 h (data not shown) and EHEC adherence was significantly enhanced by microaerobic compared with oxygenated conditions (Fig. 5A, $P = 0.0045$). SDS-PAGE and Western blot analysis of bacterial and cell lysates showed increased EspA and Tir expression on non-adherent and adherent bacteria under microaerobiosis respectively (Fig. 5B). An additional Tir band of higher molecular weight was observed in lysates from cells infected under microaerobic conditions (Fig. 5B). This represents translocated Tir that has been serine- and/or threonine-phosphorylated by host cell kinases resulting in an increase in apparent molecular weight from 72 to 88 kDa (Devinney *et al.*, 1999). Enhanced Tir translocation under microaerobic conditions was confirmed by immunofluorescence staining, which showed translocated Tir associated with $49.3 \pm 3.6\%$ of adherent EHEC under microaerobiosis compared with $15.0 \pm 2.3\%$ under oxygenated conditions (mean $\pm$ SEM, $n = 4$, $P = 0.0022$; Fig. 5C).

Type III secretion in the vertical diffusion chamber system is host cell-dependent

To examine whether the presence of host cells was required for T3S, half chambers were assembled containing either Snapwell filters with T84 monolayers or empty Snapwell support rings without cell monolayers present. Chambers without filters were perfused with oxygenated or anaerobic gas mixtures on both sides whereas chambers with cell monolayers were maintained as described above. Chambers were inoculated with lower bacterial numbers than in previous experiments to prevent medium exchange, and incubations were continued until bacteria reached early to mid-logarithmic phase ($OD_{600} = 0.2\text{--}0.3$) at approximately 3 h after inoculation. This was equivalent to the bacterial density obtained at 6 h post infection with medium replacement every 2 h in previous experiments. Whereas apical media from T84-containing chambers showed a similar pattern of secreted bacterial proteins as observed after 6 h of infection, no protein bands were observed in supernatants from chambers with EHEC only on stained protein gels (data not shown). Western blotting showed no secretion of Tir, EspA and EspB in supernatants from chambers with EHEC only whereas supernatants from T84-containing chambers showed enhanced T3S under microaerobic conditions as observed before (Fig. 6A). Interestingly, growth of non-adherent bacteria was significantly enhanced in the presence of T84 cells under both microaerobic and oxygenated conditions ($P < 0.0001$ and $P = 0.0036$ respectively; Fig. 6B). Enhanced bacterial growth and T3S appeared to be dependent on bacteria–host cell contact as no effect was observed on bacteria incubated in T84 cell-conditioned medium (apical medium obtained from non-infected T84 cells after 6 h incubation in the diffusion chamber system, data not shown).

Enhanced microaerobic colonization is mainly mediated via EspA

We further investigated whether bacterial adherence factors other than EspA contributed to increased bacterial colonization under microaerobic conditions. Therefore, infections were performed with an isogenic *espA* deletion mutant (EHEC *espA*) and its complemented strain [EHEC *espA* (*pespA*)] and numbers of adherent bacteria were determined. As shown in Fig. 7A, colonization levels of EHEC *espA* were not significantly enhanced by low oxygen levels and were similar to those of wild-type (wt) EHEC under oxygenated conditions (Fig. 7A, $P > 0.05$). In contrast, wt EHEC and EHEC *espA* (*pespA*) showed

significantly increased adherence under microaerobiosis (Fig. 7A, $P=0.0002$ and $P=0.0044$ respectively). Growth of non-adherent EHEC was significantly enhanced under oxygenated conditions in all strains tested (Fig. 7B, wt: $P=0.0048$, *espA*: $P=0.0026$ and *espA* (*pespA*): $P=0.0012$).

Influence of electron acceptors on T3S and colonization

Previous studies have shown that maturation of the EHEC T3S system under anaerobiosis requires the presence of terminal electron acceptors (Ando *et al.*, 2007). To investigate whether T3S under microaerobic conditions could be further enhanced by the presence of electron acceptors, diffusion chamber experiments were conducted in the presence of trimethylamine *N*-oxide (TMAO) or nitrate that have been shown to act as electron acceptors for anaerobic respiration in EHEC (Ando *et al.*, 2007). As shown in Fig. 8A, secretion of Tir, EspA and EspB was comparable under microaerobic conditions with or without electron acceptors. Similarly, colonization levels under microaerobic conditions were not significantly affected by the addition of TMAO or nitrate ($P>0.05$) whereas a significant reduction in colonization levels was observed under oxygenated conditions ($P<0.05$ relative to microaerobic conditions without electron acceptor or with nitrate and $P<0.01$ relative to microaerobiosis with TMAO, Fig. 8B).

Discussion

In this study we have investigated EHEC colonization in an environment simulating the microaerobic conditions in the human gut. Although accurate data about oxygen concentrations in the human intestine are still lacking, non-invasive imaging of oxygen levels in the gastrointestinal tract of living mice indicate that the milieu in the mid-small intestine and mid-colon is microaerobic (1.4% of atmospheric pressure) (He *et al.*, 1999). This is very similar to the apical oxygen concentrations generated in the vertical diffusion chamber system (1–1.7%). Polarized T84 and Caco-2 colon carcinoma cells have been chosen as model cell lines as they have been extensively used to study EHEC pathogenesis and develop high TER after polarization. Morphology and integrity of cell monolayers in the diffusion chamber was not influenced by apical microaerobic conditions during the 6 h experiment, which is consistent with Cottet and colleagues (2002) who have used a similar system with polarized Caco-2 cells to investigate *Helicobacter pylori* infection under microaerobiosis. Tight junction integrity was not affected by EHEC infection under the conditions employed [infection with stationary culture at a multiplicity of infection (moi) of 200 bacteria per cell for 6 h], which agrees with previous studies showing that EHEC causes a much slower and less pronounced decrease in TER than EPEC (Philpott *et al.*, 1998; Viswanathan *et al.*, 2004). Whereas cell morphology and EHEC colonization patterns did not differ under apical microaerobic or oxygenated conditions, adherence levels were approximately 2× higher when apical chambers were maintained under microaerobiosis. Similar diffusion chamber studies have been performed with *H. pylori* and more than 20× increased adherence levels after 4 h of infection have been reported under microaerobic conditions (Cottet *et al.*, 2002). This more dramatic effect of microaerobiosis on adherence levels is likely due to the high oxygen sensitivity of *H. pylori* that resulted in poor survival of bacteria under aerobic conditions (Cottet *et al.*, 2002). In contrast, EHEC is a facultative

anaerobe and non-adherent bacteria showed better growth at high than at low oxygen levels. *Escherichia coli* can utilize two respiratory oxidases for reduction of oxygen. Whereas the low-affinity cytochrome *bo*₃ oxidase is active under oxygen-rich conditions, the high-affinity cytochrome *bd* oxidase can still bind oxygen at low concentrations but is energetically less efficient (Gunsalus and Park, 1994; Jones *et al.*, 2007). Interestingly, an earlier study (James and Keevil, 1999) investigated growth of EHEC in steady-state chemostat cultures and yielded similar results to our diffusion chamber study: whereas growth of anaerobic cultures was impaired relative to aerobic ones, anaerobic cultures were more adhesive for HEp-2 cells.

Enhanced EHEC adherence under microaerobic conditions was associated with increased expression and secretion of T3S proteins by both adherent and non-adherent bacteria. Environmental conditions influencing EHEC or EPEC T3S have been investigated in the past and it has been shown that maximal T3S and A/E lesion formation occurs at 37°C, pH 7 and physiological osmolarity, i.e. conditions that prevail in the human intestinal tract (Rosenshine *et al.*, 1996; Kenny *et al.*, 1997b). The T3S is also enhanced in the presence of sodium bicarbonate, calcium and millimolar concentrations of Fe(NO₃)₃ (Kenny *et al.*, 1997b; Abe *et al.*, 2002) and is affected by acid stress (House *et al.*, 2009). Interestingly, only one report has investigated the impact of low oxygen concentrations on EHEC T3S so far. In the study (Ando *et al.*, 2007), EHEC were grown to early stationary phase in LB medium and supernatant proteins were examined. While good T3S was observed in cultures grown under aerobic conditions, only very low amounts of T3S proteins were detected under anaerobiosis. However, addition of alternative terminal electron acceptors (nitrate or TMAO) restored anaerobic T3S to aerobic levels and resulted in enhanced EspA filament formation and adherence to Caco-2 cells. Further experiments demonstrated that growth without electron acceptor resulted more frequently in a premature T3S apparatus lacking EspA filament and EscF needle complex (Ando *et al.*, 2007). This study prompted us to investigate the influence of terminal electron acceptors in our system. Neither addition of nitrate nor of TMAO resulted in further enhancement of T3S or colonization. In contrast to Ando and colleagues (2007), oxygen concentrations in our diffusion chamber system were microaerobic rather than anaerobic. Under these conditions, *E. coli* can still perform aerobic respiration via the high-affinity cytochrome *bd* oxidase, whereas other electron acceptors than oxygen (nitrate, nitrite, DMSO/TMAO, or fumarate) are required for respiration under anaerobic conditions (Gunsalus and Park, 1994; Jones *et al.*, 2007). Although Ando and colleagues (2007) observed good EHEC T3S under aerobic conditions, we did not detect secretion of Tir, EspA or EspB without the presence of host cells. This discrepancy may arise from differences in growth media (LB versus DMEM/F-12), bacterial growth phase (early stationary versus early to mid-logarithmic phase), or the presence of 5% CO₂ in our diffusion chamber system. While there are no further reports regarding T3S in EPEC or EHEC, a study of diffusely adhering *E. coli* (Diard *et al.*, 2006) demonstrated increased secretion of virulence associated Dr fimbriae under anaerobiosis.

In addition to environmental conditions, T3S and A/E lesion formation are dependent on growth phase, with maximal activity occurring at early to mid-logarithmic phase (OD₆₀₀ = 0.2–0.4) (Rosenshine *et al.*, 1996; Kenny *et al.*, 1997b). The T3S is also activated by quorum sensing during transition from late exponential to stationary phases (Sperandio *et al.*, 1999).

In our diffusion chamber system, we aimed to maintain bacterial growth within early to mid-logarithmic phase to avoid the effect of quorum sensing. This was achieved by exchanging apical media every 2 h, which also helped to level out bacterial growth differences in oxygenated and microaerobic apical chambers.

Another important factor contributing to T3S and bacterial growth is the presence of host cells. This has also been observed by Cottet and colleagues (2002) where growth of *H. pylori* in the microaerobic diffusion chamber system was only sustained in the presence of Caco-2 cells. As T84-conditioned medium failed to promote EHEC T3S and bacterial growth, this effect appears to be dependent on direct host cell contact. In the infection protocol, apical media were exchanged every 2 h so that the majority of non-adherent bacteria are probably derived from adhering and detaching bacteria, which continue to secrete effector proteins into the medium. Microarray studies have investigated EHEC gene transcription in response to host cell adherence. Whereas Dahan and colleagues (2004) showed downregulation of most of the LEE mRNAs at 5 h of infection of red blood cells compared with incubation in medium alone, other studies (Kim *et al.*, 2009) reported no significant difference between LEE mRNA expression at 3 h of infection of intestinal HT-29 cells and the medium control. While both of these studies focus on a particular time point of infection, kinetic real-time PCR analysis of EPEC LEE gene expression during HEp-2 cell infection showed induced *tir* and *espA* mRNA expression at 3 h, which remained constant throughout 5 h of infection (Leverson and Kaper, 2005). Inconsistent results in virulence gene expression during infection are likely to be caused by differences in host cell type but may also vary according to the infection protocol. In particular, it is difficult to achieve synchronized infection at later stages where bacteria will consist of a mixed population of attached, released and newly attaching bacteria.

Apart from EspA, which has been shown to be essential for EHEC adherence to human epithelial cells (Ebel *et al.*, 1998), no other major adherence factors appear to be involved in increased microaerobic colonization as adherence levels of an isogenic *espA* deletion mutant were not affected by low oxygen levels and were similar to those of wt EHEC under oxygenated conditions. Although previous studies have shown that EHEC flagella are involved in adherence to bovine intestinal epithelial cells (Erdem *et al.*, 2007; Mahajan *et al.*, 2009), they do not play a role in colonization of pigs (Best *et al.*, 2006) and were only expressed on very few bacteria in the diffusion chamber system independent of oxygen levels. Similar results were observed for the expression of ECP, which has been demonstrated to promote adherence to human epithelial cells (Rendón *et al.*, 2007). Interestingly, a study on sorbitol-fermenting EHEC O157:NM described induced expression of Sfp fimbriae under anaerobiosis, which resulted in increased adherence to intestinal epithelial cells (Müsken *et al.*, 2008). However, genes for Sfp fimbriae are unique for sorbitol-fermenting EHEC O157:NM and absent in O157:H7 strains (Friedrich *et al.*, 2004).

In *E. coli*, changes from aerobic to anaerobic growth and vice versa are regulated by the two global regulators Fnr (anaerobiosis) and ArcA (microaerobiosis) (Gunsalus and Park, 1994; Alexeeva *et al.*, 2003; Jones *et al.*, 2007). Whereas it has been shown that many *Salmonella* virulence genes are regulated by Fnr and thus are affected by anaerobic conditions (Fink *et al.*, 2007; Van Immerseel *et al.*, 2008), no such link has been reported for pathogenic *E. coli*

so far. Our study has shown that EHEC T3S and colonization are enhanced by microaerobiosis and it will be important to elucidate the underlying pathways involved in this phenomenon to gain a better understanding of how EHEC adapts to the microaerobic environment and successfully colonizes the human gut.

Experimental procedures

Cell culture

Human colon carcinoma T84 cells (ATCC CCL-248) were cultured in DMEM/F-12 mixture supplemented with 10% foetal bovine serum (Sigma) and used between passage 42 and 55. Human colon carcinoma Caco-2 cells (ECACC 86010202) were grown in DMEM medium supplemented with 10% foetal bovine serum and non-essential amino acids (Sigma). For diffusion chamber experiments, 5×10^5 T84 cells cm^{-2} or 2×10^5 Caco-2 cells cm^{-2} were seeded on collagen-coated polyester Snapwell filter inserts (12 mm diameter, 0.4 mm pore; Corning Costar). The TER was monitored using an EndOhm chamber and EVOM resistance meter (WPI) and values of around $1500 \Omega \times \text{cm}^2$ (T84) or $500 \Omega \times \text{cm}^2$ (Caco-2) after 10–14 days of differentiation indicated establishment of epithelial barrier function. Cells were grown at 37°C in a 5% CO_2 atmosphere.

Bacterial strains and culture conditions

The EHEC strain TUV 93-0 (Shiga toxin-negative O157:H7 EDL933), TUV 93-0 *espA* (ICC288, Shaw *et al.*, 2008), TUV 93-0 *espA* (*pespA*) (ICC288 complemented with pBAD-EspA EHEC O157:H7, Crepin *et al.*, 2005) and the non-pathogenic *E. coli* strain HB101 were obtained from G Frankel (Imperial College London). For infections, bacteria were grown standing in Brain Heart Infusion broth overnight at 37°C . Ampicillin ($100 \mu\text{g ml}^{-1}$) and kanamycin ($50 \mu\text{g ml}^{-1}$) were added as required.

Infection in a vertical diffusion chamber system

Snapwell filter inserts with polarized cells were mounted between two half chambers of a vertical diffusion chamber system (Harvard Apparatus). Both compartments were filled with serum-free culture medium and 10^8 bacteria (moi of 200) were added to the apical side (Fig. 1). Apical chambers were perfused with 95% O_2 , 5% CO_2 (oxygenated) or 90% N_2 , 5% H_2 , 5% CO_2 (anaerobic) whereas basolateral compartments were kept under oxygenated conditions. Oxygen concentrations in apical compartments were monitored during the experiment using an ISO2 dissolved oxygen meter (WPI). Apical media were exchanged every 2 h to maintain bacteria at early exponential growth phase and incubations were continued for 6 h. For electron acceptor studies, 10 mM sodium nitrate or TMAO (Sigma) was added to the apical chamber. To investigate the influence of host cells on T3S, parallel diffusion chamber experiments were performed with or without cell monolayers. Bacteria were inoculated at a lower concentration (2.5×10^7 , moi of 50) to prevent medium exchange. Incubations were carried out for approximately 3 h when bacteria had reached early exponential phase ($\text{OD}_{600} = 0.2\text{--}0.3$), which was similar to bacterial densities at 6 h post infection with 2 h medium exchange. At the end of the experiment, apical media were collected and OD_{600} values were determined to assess growth of non-adherent bacteria. Bacteria were pelleted by centrifugation and supernatants and pellets were stored at -20°C .

for SDS-PAGE analysis. Membrane filters were cut out from supports, washed twice in cold PBS to remove non-adherent bacteria and processed according to further analysis.

SEM

Filters were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% aqueous osmium tetroxide and dehydrated in 2,2 dimethoxy-propane. Specimens were transferred to absolute ethanol, critically point-dried using liquid carbon dioxide (Emitech K850 apparatus), mounted on aluminium stubs, sputter-coated with gold-palladium (Polaron E5100 sputter coater) and viewed in a JEOL 5300 SEM.

Immunofluorescence staining and microscopic quantification

Filters were fixed in formalin for 10 min and blocked/permeabilized with 0.1% Triton X-100 (Tx-100), 0.5% BSA in PBS for 20 min. For occludin staining, cells were pre-extracted with 0.2% Tx-100 in PBS for 2 min on ice, fixed in formalin and permeabilized with 0.05% Tx-100 in PBS for 5 min on ice as recommended by the manufacturer. Cells were subsequently incubated in primary antibodies (goat anti-O157:H7 from Fitzgerald Industries; rabbit anti-occludin from Invitrogen; rabbit anti-Tir and anti-EspA from G Frankel, Imperial College London; rabbit anti-ECP from JA Girón, University of Florida, USA) for 60 min, washed and incubated in Alexa Fluor-conjugated anti-goat or anti-rabbit IgG (Invitrogen) for 30 min. Cell nuclei/bacterial DNA were labelled with propidium iodide (Sigma) or DAPI (Roche). Filters were mounted in Vectashield (Vector Laboratories) and analysed using a fluorescence light microscope (Axio Imager, Zeiss) or confocal laser scanning microscope (LSM 510 Meta, Zeiss). To quantify ECP and flagella expression and Tir translocation, 50 randomly chosen infected cells were examined and at least 200 adherent bacteria were counted in three separate experiments.

Quantification of adherent bacteria

Cell monolayers on filters were lysed in 1% Tx-100 in PBS for 30 min on ice. Serial dilutions were plated out on LB agar plates and cfu were determined after overnight incubation at 37°C.

SDS-PAGE and Western blot analysis of T3S proteins

The EHEC lysates were prepared by suspending bacterial pellets in reducing SDS-PAGE sample buffer. Supernatant proteins were precipitated by addition of 10% trichloroacetic acid (v/v) for 1 h at 4°C. Precipitated proteins were pelleted by centrifugation at 4°C (16 000 g, 15 min) and suspended in reducing sample buffer. For cell lysates, polarized monolayers were lysed in ice-cold RIPA buffer containing 1 mM PMSF, 1 mM Na₃VO₄ and protease inhibitor cocktail (1:200, Sigma). Sample volumes of bacterial lysates and precipitated proteins were adjusted according to bacterial density (OD₆₀₀) and resolved on 12% SDS-polyacrylamide gels. Proteins were visualized by Instant Blue staining (Triple Red). For Western blotting, proteins were transferred to nitrocellulose (Hybond, GE Healthcare). Membranes were blocked with 3% BSA in TBS/0.05% Tween-20 for 60 min and incubated with primary antibodies (rabbit anti-Tir, EspA and EspB from G Frankel, Imperial College London; rabbit anti-GroEL from Sigma) overnight at 4°C. After washing, blots were

incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) and developed using enhanced chemiluminescence (Immobilon Western, Millipore).

Statistics

Statistical analysis was performed using GraphPad prism software. Student's paired *t*-test was used to determine differences between two groups; one-way ANOVA with Tukey-Kramer's multiple comparison post test was used for multiple groups. A *P*-value of < 0.05 was considered significant.

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Fig. 1.

Infection in a vertical diffusion chamber system. Polarized intestinal epithelial cells grown on Snapwell filters were inserted between two half chambers and infected apically with EHEC. Apical chambers were perfused with 95% O₂, 5% CO₂ (oxygenated) or 90% N₂, 5% H₂, 5% CO₂ (anaerobic) whereas basolateral compartments were maintained under oxygenated conditions.

Fig. 2.

Cell morphology and bacterial adherence patterns of polarized T84 cells infected with EHEC for 6 h under apical microaerobic or oxygenated conditions. Shown are representative images from three independent experiments.

A. Confocal micrographs of non-infected (NI) and infected monolayers (EHEC) stained for occludin (green). Bacteria and cell nuclei were stained with propidium iodide (magenta). Bars = 10 μm.

B. Scanning electron micrographs of non-infected and infected T84 cells. Bars = 1 μm.

Fig. 3. Microaerobic apical conditions enhance EHEC colonization (A), but impair growth of non-adherent bacteria (B). T84 cells were infected with EHEC for 6 h and maintained under apical oxygenated (OX) or microaerobic conditions (MA). Adherent bacteria were quantified as cfu/filter (A) whereas growth of non-adherent bacteria was assessed by OD₆₀₀ (B). Data are shown as means ± SEM from four independent experiments performed in triplicate. *** $P < 0.001$.

Fig. 4. Apical microaerobic conditions enhance expression and secretion of EHEC T3S proteins. A. Stained SDS-PAGE of EHEC-secreted proteins under oxygenated (OX) or microaerobic conditions (MA). Differentially secreted proteins are marked by asterisks and molecular weights are indicated in kDa. B. Western blot of EHEC-secreted proteins probed with Tir, EspA and EspB specific antisera.

C. Western blot of EHEC and T84 cell lysates showing Tir and EspA expression by non-adherent (NA) and adherent (A) bacteria respectively. Cell lysates of non-infected (NI) T84 monolayers were included to confirm specificity of antisera. Relative amounts of loaded bacterial protein were assessed by GroEL immunoblotting.

D. Immunofluorescence staining of EHEC adhering to T84 cells after 6 h of infection showing enhanced EspA filament formation (green) under microaerobic compared with oxygenated conditions (first two panels on the left). In contrast, expression of ECP (green, second panel from the right) and flagella (red, panel on far right) was not affected by oxygen levels. EHEC were labelled with anti-O157:H7 (red) and cellular/bacterial DNA was counterstained with DAPI (blue, far right panel only). Shown are representative images from three independent experiments.

Fig. 5.

A. EHEC adherence to polarized Caco-2 cells is elevated under microaerobic conditions. Data are shown as means $\pm$ SEM from four independent experiments performed in triplicate. ** $P < 0.01$.

B. Western blot of EHEC and Caco-2 cell lysates showing Tir and EspA expression by non-adherent (NA) and adherent (A) bacteria respectively. Translocated Tir is indicated by an asterisk. Relative amounts of loaded bacterial protein were assessed by GroEL immunoblotting.

C. Immunofluorescence staining of polarized Caco-2 cells infected with EHEC for 6 h. Translocated Tir (green) is evident beneath adherent bacteria (propidium iodide, red).

Fig. 6. EHEC T3S and growth of non-adherent bacteria are host cell-dependent. (A) Western blot of bacterial supernatant proteins probed with Tir, EspA and EspB specific antisera and (B) OD₆₀₀ of apical media. Bacteria were grown under microaerobic (MA) or oxygenated conditions (OX) in the absence (-T84) or presence of T84 cells (+T84).
 A. Protein samples were run on two separate gels that were processed simultaneously.
 B. Data are shown as means $\pm$ SEM from two independent experiments performed in duplicate. ** $P < 0.01$, *** $P < 0.001$.

Fig. 7.

Enhanced microaerobic adherence of EHEC to polarized T84 cells is mainly dependent on EspA. T84 cells were infected with wt EHEC, an isogenic *espA* mutant, and its complemented strain [Δ (*pespA*)] for 6 h while maintained under apical oxygenated (OX) or microaerobic conditions (MA). Adherent bacteria were quantified as cfu/filter (A) whereas growth of non-adherent bacteria was assessed by OD₆₀₀ (B). Data are shown as means $\pm$ SEM from four independent experiments performed in triplicate. ** $P < 0.01$, *** $P < 0.001$.

Fig. 8.

Addition of nitrate or TMAO as terminal electron acceptors does not further enhance EHEC T3S or colonization under microaerobic conditions. T84 cells were infected with EHEC for 6 h under apical oxygenated (OX) or microaerobic (MA) conditions. Nitrate (N) or TMAO (T) was added to apical chambers.

A. Western blot of bacterial supernatant proteins probed with anti-Tir, EspA and EspB.

B. Colonization of T84 cells by EHEC. Data are shown as means $\pm$ SEM from two independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$.