Cell invasion and survival of Shiga toxin-producing
*Escherichia coli* within cultured human intestinal epithelial cells

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Shiga toxin-producing *Escherichia coli* (STEC) cause severe human infections and their virulence abilities are not fully understood. Cattle are a key reservoir, and the terminal rectum is the principal site of bacterial carriage. Most STEC possess a pathogenicity island termed the locus of enterocyte effacement (LEE). Nonetheless, LEE-negative STEC have been associated with disease. We found that invasion of LEE-positive and LEE-negative strains was higher for human enterocytic cell lines and for undifferentiated Caco-2 cells. Intracellular bacteria could be detected as early as 5 min after infection and transmission electron microscopy showed bacteria within membrane-bound vacuoles. STEC invasion depended on actin microfilaments and protein kinases. Scanning electron microscopy revealed that bacterial entry was not associated with membrane ruffling. Absence of macropinocytosis or actin rearrangement at the entry points suggests a zipper-like entry mechanism. Disruption of the tight junction by EGTA enhanced invasion of Caco-2 monolayers, and bacterial invasion mostly proceeded through the basolateral pole of enterocytes. STEC persisted within Caco-2 cells for up to 96 h without cell death and bacterial viability increased after 48 h, suggesting intracellular multiplication. The relatively harmless intracellular localization of STEC can be an efficient strategy to prevent its elimination from the bovine intestinal tract.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are emergent pathogens implicated in foodborne outbreaks and severe human infections worldwide. These microorganisms colonize the human gut and can cause diseases ranging from self-limited watery diarrhoea to haemorrhagic colitis and the life-threatening sequelae known as haemolytic uraemic syndrome. Cattle are the main natural reservoir for STEC and the human infection can be foodborne as well as transmitted via other humans, direct or indirect contact with animals or through contaminated water (Paton & Paton, 1998; Caprioli et al., 2005). Shiga toxin is the main virulence feature of STEC but other bacterial properties such as adherence to and colonization of the gut are also important. Highly virulent serotypes such as O157:H7 are capable of colonizing the intestinal mucosa with an attaching-and-effacing (A/E) mechanism, genetically governed by the chromosomally encoded pathogenicity island termed locus of enterocyte effacement (LEE). LEE contains genes for a type III protein secretion system, an array of effector proteins translocated into host cells and an adherence system consisting of an outer-membrane protein ( intimin) and its translocated intimin receptor (Tir). A/E lesions are characterized by localized destruction of brush border microvilli, intimate bacterial adhesion and formation of actin pedestals beneath the attached bacteria (Kaper et al., 2004).

Although most STEC strains isolated from patients are A/E pathogens, LEE-negative serotypes such as O113:H21, O91:H21, O8:H19, O121:H19 and O104:H4 have been associated with sporadic and outbreak cases of severe disease indistinguishable from that caused by other LEE-positive STEC (Caprioli et al., 2005; Karch et al., 2005; Scheutz et al., 2011).

Additional virulence factors have been described, mostly putative adhesins and cytotoxins. Their genes reside in

**Abbreviations:** A/E, attaching-and-effacing; EPEC, enteropathogenic *E. coli*; LEE, locus of enterocyte effacement; MTT, 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide; SEM, scanning electron microscopy; STEC, Shiga toxin-producing *Escherichia coli*; TEM, transmission electron microscopy.

Four supplementary figures and a supplementary table are available with the online version of this paper.
mobile genetic elements such as plasmids, phages and pathogenicity islands and were found both in LEE-negative and LEE-positive STEC strains (Paton & Paton, 1998; Caprioli et al., 2005; Lim et al., 2010).

Concerning the invasive ability of STEC, early studies carried out with O157:H7 strains have yielded conflicting results, against (McKee & O’Brien, 1995) or in favour, supporting the invasion of certain cell lines (Oelschlaeger et al., 1994) and free-living amoebae (Barker et al., 1999). Luck et al. (2005) showed that LEE-negative O113:H21 strains were more invasive than O157:H7 strains in CHO-K1 (Chinese hamster ovary) cells.

Previous studies showed that the recto-anal junction mucosa is the primary site of O157:H7 colonization in cattle (Grauke et al., 2002; Naylor et al., 2005; Cobbold et al., 2007; Dean-Nystrom et al., 2008), and the carriage of the micro-organism at this site is correlated with high-level faecal shedding (Stevens et al., 2002; Low et al., 2005). Recently, work seeking to explain the bacterial colonization of the bovine intestinal mucosa by STEC moved from the role played by bacterial adhesins to the investigation of STEC invasiveness (Sheng et al., 2011).

In this work, we intend to compare STEC strains carrying or not carrying the LEE pathogenicity island regarding their ability to invade human epithelial cells from enterocytic (Caco-2, T-84) and non-enterocytic lineages (HeLa, HEp-2), and study cell entry as a function of the differentiation status of the intestinal cell, as well as the bacterial survival and persistence in the intracellular compartment of the cultured human enterocyte cell lineage Caco-2.

METHODS

Bacteria and growth conditions. The bacterial strains used in this study are listed in Table 1. Bacteria were grown statically in TSB (tryptic soy broth; Difco) for 18–20 h at 37 °C, centrifuged and resuspended in D-PBS (Dulbecco’s PBS; pH 7.2 Invitrogen). Bacterial concentrations were determined by densitometry and plating on TSA (tryptic soy agar; Difco).

Human cell lines and culture conditions. Caco-2 cells (ATCC HTB37; colon adenocarcinoma) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma), containing 10% (v/v) FCS (Invitrogen), 1% (v/v) non-amino acids mixture (Invitrogen), 4 mM l-glutamine (Invitrogen) and antibiotics (50 μg gentamicin ml⁻¹; 2.5 μg fungazole ml⁻¹). Caco-2 cells spontaneously differentiate in culture upon reaching post-confluence conditions (12 days). Subconfluent cultures (5 days) produce cell islets in which peripheral cells are non-polarized and undifferentiated. Cell differentiation status was assessed by indirect immunofluorescence staining of the brush border-associated marker sucrase-isomaltase, as previously described (Rosa et al., 2001), and cell viability was monitored with the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Denizot & Lang, 1986). T-84 cells (ATCC CCL248; colon carcinoma) were grown for 14 days in DMEM/F-12 medium (Invitrogen) supplemented with 10% (v/v) FCS, 25 mM HEPES, 4 mM l-glutamine and antibiotics. HEP-2 (ATCC CCL23; larynx carcinoma) and HeLa (ATCC CCL2; cervix carcinoma) cells were grown for 48 h in DMEM supplemented with 5% (v/v) FCS and antibiotics. Cells were incubated under 5% CO₂/95% air at 37 °C.

Quantitative invasion assay. The bacterial invasion was measured as protection against amikacin killing (Rosa et al., 2001). Experiments were run in triplicate and repeated at least three times. All strains were found to be susceptible to amikacin (MIC 1–5 μg amikacin ml⁻¹). Bacterial viability was unaffected after incubation (1 h at 37 °C) with 1% (v/v) Triton X-100. Salmonella Typhimurium C20 and E. coli C600 (K12) were used as positive and negative controls, respectively. Caco-2 monolayers grown for 5 and 12 days were used for comparing bacterial invasiveness between undifferentiated and differentiated Caco-2 cells.

Disruption of intercellular junctions by EGTA and bacterial invasion of basolateral cell domain. Fully differentiated Caco-2 monolayers were washed with Ca²⁺/Mg²⁺-free D-PBS and incubated with Ca²⁺/Mg²⁺-free D-PBS supplemented with 0.1 M EGTA (Sigma) for 1 h prior to infection. Control monolayers were incubated in D-PBS. Thereafter, monolayers were washed once and overlaid with DMEM (supplemented with 2%, v/v, FCS and 55 mM D-mannose) and the quantitative invasion assay was performed for 2 h. Shigella flexneri and S. Typhimurium were used as controls.

Survival of bacteria within Caco-2 cells. Undifferentiated Caco-2 monolayers were infected with 10⁸ bacteria and the tissue-culture plates were centrifuged (730 g) for 10 min to optimize bacteria–cell interaction. After a 1 h incubation, 250 μg amikacin ml⁻¹ was added for 1 h to kill extracellular bacteria. Cell monolayers were then incubated with fresh medium with 10% (v/v) FCS and 10 μg amikacin ml⁻¹ for 6, 24, 48, 72 and 96 h. At each time point cells were washed, lysed and the cell lysates were diluted and plated on TSA. Viability of Caco-2 cells was monitored with the MTT assay.

Intracellular live and dead bacteria within Caco-2 cells. Live and dead bacterial populations within Caco-2 cells were determined according Buchmeier & Libby (1997). Briefly, cells were grown in six-well tissue-culture plates and the experiment was performed as described for the intracellular survival assay. At each time point the infected monolayers were washed and bacteria were harvested by lysing cells with 12 mM sodium deoxycholate (Sigma) for 30 min. The lysate was centrifuged and the pellet was recovered. The live/dead kit reagents were combined as instructed by the manufacturer (BacLight Live/Dead kit; Molecular Probes) and added to the pellet, to label both live and dead bacteria simultaneously. Bacteria were visualized using a Zeiss Axioplan 2 fluorescence microscope and percentages of live (green) and dead (red) bacteria were recorded.

Effects of eukaryotic cell inhibitors in bacterial invasion. For assessing the effects of the cytoskeletal inhibitors 2 μg cytochalasin D ml⁻¹ (Sigma) and 5 μg colchicine ml⁻¹ (Sigma) and the protein kinase inhibitors 0.5 μg staurosporine ml⁻¹ (Sigma) and 27 μg genistein ml⁻¹ (ICI) on bacterial adherence and internalization. Caco-2 cells were treated with the drugs for 30 min (genistein, 10 min) prior to addition of bacteria. Stock solutions of inhibitors were prepared in DMSO (Sigma). The quantitative invasion test was performed as described above (Rosa et al., 2001), and the inhibitors were present throughout the invasion assay. The effect of each drug was evaluated against a control assay without the drug, which defined a 100% invasion. S. Typhimurium C20 and enteropathogenic E. coli (EPEC) strain E2348/69 were used as controls. Cell and bacterial viability following 4 h of incubation in medium containing the highest concentration of inhibitors and DMSO was assessed by the MTT assay and viable counts respectively.

Microscopy. Undifferentiated monolayers of Caco-2 cells were prepared on circular 13 mm glass coverslips in 24-well tissue culture plates. Cells were infected with 10⁵ bacteria and incubated for 5, 15, 30 and 60 min and 3 h. At each time point, cell monolayers were washed twice with D-PBS and fixed with methanol (for Giemsa’s
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Relevant characteristics*</th>
<th>Source†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LEE-positive STEC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0651/1</td>
<td>O103:H2</td>
<td>stx1, eae, E-hlyA</td>
<td>Human disease, Brazil</td>
</tr>
<tr>
<td>2781/8</td>
<td>O111,a,c:NM</td>
<td>stx1, eae θ, E-hlyA, astA, iha</td>
<td>Human disease, Brazil</td>
</tr>
<tr>
<td>183/1</td>
<td>O153:H25</td>
<td>stx1, eae NT, E-hlyA, espP</td>
<td>Healthy cattle, Brazil</td>
</tr>
<tr>
<td>EDL 931 (ATCC 35150)</td>
<td>O157:H7</td>
<td>stx1, stx2, eae γ, E-hlyA, lpfA_O1137/OI-141, lpfA_O1137/OI-45</td>
<td>Human disease, USA</td>
</tr>
<tr>
<td>EDL 933 (ATCC 700927)</td>
<td>O157:H7</td>
<td>stx1, stx2, eae γ, E-hlyA, lpfA_O1137/OI-141, lpfA_O1137/OI-45</td>
<td>Food, USA</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>282/2</td>
<td>O8: H19</td>
<td>stx1, stx2, E-hlyA, espP, iha, saa</td>
<td>Healthy cattle, Brazil</td>
</tr>
<tr>
<td>637/1</td>
<td>O22 : H8</td>
<td>stx1, iha</td>
<td>Healthy cattle, Brazil</td>
</tr>
<tr>
<td>208/2</td>
<td>O105:NM</td>
<td>stx1, stx2, E-hlyA, espP, iha, saa</td>
<td>Healthy cattle, Brazil</td>
</tr>
<tr>
<td>397/2</td>
<td>O113 : H21</td>
<td>stx1, stx2, astA, iha, lpfA_O113</td>
<td>Healthy cattle, Brazil</td>
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<td>784/1</td>
<td>O113 : H21</td>
<td>stx2, E-hlyA, espP, iha, saa, pilS, lpfA_O113, subA</td>
<td>Food, Brazil</td>
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<td><strong>Control strains</strong></td>
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<td></td>
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<tr>
<td>C600</td>
<td>E. coli K12</td>
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<td>Human disease, UK (CPHL)</td>
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<tr>
<td>E 2348/69</td>
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<td></td>
<td>Human disease, Brazil</td>
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<tr>
<td>C20</td>
<td>S. Typhimurium</td>
<td></td>
<td>Human disease, Brazil</td>
</tr>
<tr>
<td>FCF 398</td>
<td>Y. enterocolitica bioserogroup 4/O:3; invA</td>
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<td>Human disease, Brazil</td>
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<tr>
<td>1225/53</td>
<td>S. flexneri serotype 3</td>
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<td>Human disease, Brazil</td>
</tr>
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</table>

*Genes: stx1, stx2, stx2c: Shiga toxin; eae: intimin; e, γ, θ, NT; intimin types; E-hlyA: enterohaemolysin; astA: EAST-1; espP: extracellular serine-protease; saa: plasmid-mediated adhesin; iha: chromosomal adhesin; pilS: type IV pili; lpfA_O113, lpfA_O1137/OI-141, lpfA_O1137/OI-45: long polar fimbriae; subA: putative adhesin.
†Brazilian STEC strains from food or cattle were isolated by us (1999–2000) and those from human disease were kindly donated by B.E.C. Guth (UNIFESP).

staining) or 2% (w/v) paraformaldehyde (Merck) in D-PBS. Macropinocytosis detection by fluorescence microscopy was performed according Garcia-del Portillo & Finlay (1994). Briefly, cells were infected for 1 h and 3 h in fresh medium with 0.5 μg dextran-FITC ml⁻¹ (MW 10 000; Sigma), washed and fixed with paraformaldehyde. S. Typhimurium C20 and Yersinia enterocolitica FCF398 were used as positive and negative controls, respectively. For double staining of actin and bacteria, infected cells were permeabilized with 0.1% (v/v) Triton X-100, washed, fixed with 2.5% (v/v) glutaraldehyde (Sigma) in D-PBS, treated with 5% (w/v) BSA in D-PBS (blocking buffer) and incubated with rabbit antibodies (for Y. enterocolitica, S. Typhimurium or STEC) diluted 1:50 in PBS-BSA. Bacteria and cells were then stained with Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) diluted 1:200 in 1% (v/v) BSA in D-PBS (blocking buffer) plus Alexa 488-labelled phalloidin (Molecular Probes) and counterstained with DAPI (Molecular Probes). For β1-integrin labelling, mouse monoclonal BV7 antibody (1:100; Abcam) and Alexa 488-conjugated anti-mouse IgG (1:200; Molecular Probes) were used. For differential staining of intracellular and extracellular STEC, cells were treated with blocking buffer and incubated with O8 or O157 rabbit antiserum diluted 1:50 in PBS-BSA for 1 h. After washing, the cells were incubated for 1 h with Alexa 594-conjugated goat anti-rabbit IgG (Molecular Probes) diluted 1:200. Cells were permeabilized with Triton X-100, washed, treated with blocking buffer, incubated with primary antibodies, stained with Alexa 488-conjugated goat anti-rabbit IgG 1:200 and counterstained with DAPI. Coverslips were washed and mounted onto glass slides with ProLong Gold (Molecular Probes), examined in a Zeiss Axioplan 2 microscope and images were acquired using a Zeiss AxioCam MR3 camera and processed on Photoshop software.

Transmission (TEM) and scanning (SEM) electron microscopy. For TEM, undifferentiated Caco-2 cell monolayers were infected, washed, fixed with 2.5% (v/v) glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2) for 1 h at 4 °C, post-fixed in 1% (v/v) osmium tetroxide (Sigma) for 1 h, dehydrated in ethanol and embedded in Epon (EMBed-812; EMS). Ultra-thin sections were counterstained with 5% (w/v) uranyl acetate and 2.5% (w/v) lead citrate and examined in an EM 906 Zeiss microscope at 80 kV. For SEM, the cell monolayers were infected, washed and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) plus 2.5 mM CaCl₂ for 1 h at 4 °C. Cells were post-fixed with osmium tetroxide, dehydrated in acetone and critical point dried. Mounted specimens were sputter coated with gold and viewed on an LEO 1450 VP microscope operated at 20 kV.

Statistics. Results are presented as means±SD. Repeated measures ANOVA and Tukey’s post-hoc tests were used to compare invasion results between groups. Differences between two results were tested by an unpaired, two-tailed t test. A P value <0.05 was considered significant.

RESULTS

STEC invasive ability

The LEE-positive and LEE-negative strains used in this study carry distinct gene profiles for Shiga toxin, enterohaemolysin, intimin and putative adhesins. Strain

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208/2 (O105:NM) was included because previous work showed its inability to invade Caco-2 cells (Table 1).

We found that most STEC, carrying or not carrying the LEE locus, were able to attach to the cells and especially to those of enterocytic lineage. The LEE-positive strains' ability to form micro-colonies on the cells does not result in greater adherence to the cell monolayers. However, concerning STEC invasiveness, greater values were found for enterocytic cell lines, most strains showing invasion indexes 10- to 100-fold higher than those obtained for the non-enterocytic cell lines tested (Table 2 and Table S1, available in Microbiology Online). Prototype strain EDL933 (O157:H7) adhered quite efficiently to enterocytic cells, but most of the cell-associated bacteria remained in an extracellular location. The other O157:H7 strains tested including EDL931 (O157:H7), isolated from a haemorrhagic colitis case in the same outbreak as EDL933 (Riley et al., 1983), behaved as the non-O157 STEC strains tested, invading efficiently both Caco-2 and T-84 cells. Strain 2781/8 (O111:NM), although attaching efficiently to both enterocytic cell lines, was also found to invade the T-84 cells only.

Giemsa-stained post-confluent Caco-2 cell monolayers infected by STEC showed dispersed individual bacteria over the fully differentiated cells, while most LEE-positive strains also produced small microcolonies. Subconfluent (5-day-old) monolayers of Caco-2 cells formed circular islets in which peripheral cells were undifferentiated and proliferative, whereas central cells became polarized and differentiated (Fig. 1a). This cell assay system has been used extensively to study the interaction between micro-organisms and the apical and basolateral membranes of Caco-2 cells (Finlay & Falkow, 1997). Invasive STEC were observed to show a preferential association with the outer edges of cells located on the islet periphery (Fig. 1b). Some bacteria in the clusters were surrounded by a clear halo, suggesting intracellular location. LEE-positive STEC, besides interacting with the peripheral cells, also displayed small microcolonies, mostly on the differentiated central cells, especially in the later stages (3–6 h) of infection. Non-invasive STEC strain 208/2 (O105:NM) did not show a preferential cell-type association, or evidence of vacuole production (Fig. S1).

Using the quantitative invasion assay, STEC interaction was compared with undifferentiated and differentiated Caco-2 cells. For most STEC, no significant differences in bacterial adherence were detected. However, certain strains adhered more efficiently to differentiated cells (EDL933, 208/2) and others to undifferentiated cells (2781/8, 282/2, 637/1) (data not shown). No correlation was found between higher association indexes to Caco-2 or T-84 cells and the STEC profiles of putative adhesins.

Concerning bacterial invasiveness, most STEC were found to invade undifferentiated Caco-2 cells more efficiently (Fig. 1c). The exceptions were strain 0651/1 (O103:H2) that showed greater invasion in differentiated cells and

<table>
<thead>
<tr>
<th>Strain/serotype</th>
<th>Caco-2 Association*</th>
<th>Caco-2 Invasion†</th>
<th>T-84 Association</th>
<th>T-84 Invasion</th>
</tr>
</thead>
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<tr>
<td><strong>LEE-positive STEC</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0651/1 O103:H2</td>
<td>7.3 ± 3.9</td>
<td>1.2 ± 0.2</td>
<td>13.0 ± 0.2</td>
<td>1.1 ± 0.3</td>
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<tr>
<td>2781/8 O111:NM</td>
<td>10.0 ± 7.8</td>
<td>0.6 ± 0.1</td>
<td>11.0 ± 1.2</td>
<td>6.6 ± 0.6†</td>
</tr>
<tr>
<td>183/1 O153:H25</td>
<td>0.7 ± 0.4</td>
<td>6.8 ± 1.0‡</td>
<td>9.5 ± 1.5‡</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>2004/1 O157:H7</td>
<td>4.3 ± 0.9</td>
<td>5.8 ± 0.2‡</td>
<td>14.0 ± 1.5‡</td>
<td>2.3 ± 0.9</td>
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<td>6.7 ± 1.1</td>
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<td>EDL 933 O157:H7</td>
<td>29.0 ± 22.2</td>
<td>0.3 ± 0.1</td>
<td>13.4 ± 1.9</td>
<td>0.7 ± 0.6</td>
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<tr>
<td><strong>LEE-negative STEC</strong></td>
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<tr>
<td>282/2 O8: H19</td>
<td>3.0 ± 0.2</td>
<td>3.6 ± 0.4</td>
<td>2.4 ± 1.1</td>
<td>9.4 ± 1.9‡</td>
</tr>
<tr>
<td>637/1 O22: H8</td>
<td>0.9 ± 0.1</td>
<td>2.3 ± 0.5</td>
<td>14.0 ± 11.4</td>
<td>1.8 ± 0.6</td>
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<tr>
<td>208/2 O105:NM</td>
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<td>3.9 ± 2.7</td>
<td>0.5 ± 0.1‡</td>
</tr>
<tr>
<td>397/2 O113:H21</td>
<td>3.1 ± 0.2</td>
<td>3.4 ± 0.4</td>
<td>9.0 ± 6.0</td>
<td>6.5 ± 0.8‡</td>
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<td>784/1 O113:H21</td>
<td>6.0 ± 3.5</td>
<td>5.0 ± 0.2</td>
<td>50.0 ± 1.4‡</td>
<td>4.5 ± 1.6</td>
</tr>
<tr>
<td><strong>Control strains</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>S. Typhimurium C20</td>
<td>3.9 ± 0.1</td>
<td>13.0 ± 1.1</td>
<td>12.0 ± 1.1‡</td>
<td>16.0 ± 3.4</td>
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<td>E. coli C600</td>
<td>1.3 ± 1.0</td>
<td>0.0</td>
<td>6.1 ± 0.0‡</td>
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</tr>
</tbody>
</table>

*Association index: (IC + EC)/10^7 bacteria (inocula) × 100.
†Invasion index: IC/(EC + IC) × 100; IC, intracellular bacteria; EC, extracellular bacteria; significant differences (P<0.05) in bacterial association or invasion of indicated strains for Caco-2 versus T-84.
‡Mean ± SD of three independent tests, in triplicate.
2781/8 (O111 : NM), which had an invasive ability equally discreet in both situations. Cell invasion by strain 784/1 (O113 : H21), although higher with undifferentiated cells, was not statistically significant ($P < 0.070$).

**Kinetics of early STEC invasion of Caco-2 cells**

The quantitative invasion assay, performed with subconfluent Caco-2 monolayers, was used to assess the kinetics of STEC invasion in 5, 15, 30, 60 and 180 min infection periods (Fig. 2a). Bacterial adherence was found to begin swiftly and, within 5 min of infection, 0.2 to 0.6 % of the challenging inoculum ($10^7$ bacteria) were cell-associated. Cell invasion was also detected within 5 min of infection, and within 15 min of infection the intracellular bacterial counts reached 0.2 to 0.4 % of the cell-associated bacteria (data not shown). The intracellular bacterial count grew steadily with time, from $10^2$ bacteria to $10^3$ to $10^4$ bacteria in 180 min of infection. This pace is 20–200-fold higher than the increase of the cell-associated bacterial population, suggesting intracellular multiplication instead of cell entry of surface-attached bacteria alone.

**Effects of eukaryotic cell inhibitors on STEC invasion of Caco-2 cells**

Quantitative invasion assays were performed in the presence of the drugs cytochalasin D (an actin microfilament inhibitor), colchicine (inhibits the eukaryotic microtubule function), staurosporine (inhibitor of several protein kinases) and genistein (a tyrosine phosphokinase inhibitor) (Fig. 2b). *S. Typhimurium and EPEC E2348/69 were used as controls. As expected, *S. Typhimurium invasion was not affected by treating cells with colchicine, staurosporine or genistein whereas EPEC had its invasion decreased by all inhibitors tested. Invasion of STEC strains 282/2, 784/1, EDL931 and 0651/1 was significantly affected by cytochalasin D ($50.0 \pm 0.9, 42.0 \pm 0.7, 4.0 \pm 0.3$ and $56.0 \pm 5.2$ respectively) and staurosporine ($16.6 \pm 1.5, 14.0 \pm 1.0, 38.0 \pm 0.4$ and $29.0 \pm 9.8$ respectively). Caco-2 treatment with colchicine resulted in a significant decrease of invasion by LEE-positive strains EDL931 and 0651/1 ($50.0 \pm 5.2$ and $50.0 \pm 0.6$) but not for the LEE-negative strains tested, whereas genistein only inhibited invasion of EDL931 ($50.0 \pm 0.6$). Cell treatment with inhibitors affected neither bacterial or cell viability nor bacterial adherence to Caco-2 cells (data not shown).

**Internalization of STEC**

Caco-2 invasion by strains EDL931 and 282/2 was followed using double immunofluorescence to distinguish between intracellular and extracellular bacteria. After 5 min of infection,
isolated intracellular bacteria could already be seen on the undifferentiated Caco-2 cell’s outer edges from cell islets (Fig. 2c). After 1 h and 3 h of infection, intracellular bacteria increased, forming compact groups. Additional images of STEC internalization as well as absence of invasion by the non-invasive STEC 208/2 are included (Fig. S2).

**Fig. 2.** STEC invasion of undifferentiated Caco-2 cells. (a) Kinetics of early STEC invasion. Data represent intracellular bacterial counts, as determined by the amikacin-killing assay. Invasion in 5 min was not detected for STEC strain EDL 931. Bars, mean ± SD from three independent experiments in triplicate. Error bars are not apparent if <0.1 (b). Effects of cytochalasin D (2 μg ml⁻¹), colchicine (5 μg ml⁻¹), staurosporine (0.5 μg ml⁻¹) and genistein (27 μg ml⁻¹) on Caco-2 invasion (3 h) by STEC strains and control bacteria (S. Typhimurium C20 and EPEC E2348/69). Invasion was determined by the amikacin-killing assay. Results as per cent invasion are means ± SD from three separate experiments. *P<0.05 versus respective control. (c) Merged images of phase-contrast and double immunofluorescence labelling of extracellular (red) and intracellular (green) STEC infecting undifferentiated, non-polarized cells after 5 min, 1 h (strain 282/2; O8 : H19) and 3 h (strain EDL931; O157 : H7) post-infection. Even for 5 min of infection, most of the cell-associated bacteria are intracellularly located. Bar, 5 μm. (d) TEM of Caco-2 cell infected (6 h) by STEC 784/1 (O113 : H21). Bacterial cells are enclosed within vacuoles whose membrane appears intact and bacteria are frequently associated with it (arrowheads). (e) SEM of STEC 784/1 entry (30 min), with membrane sheets tightly wrapped around bacteria (arrowheads). The shapes of internalized bacteria can be seen (*). Membrane projections, folds and ruffles in the host cell membrane are absent. (f) SEM of STEC 784/1 entry (30 min). A fully internalized bacterial group, homogeneously recovered by cell membrane. The shapes of the protuberant bacterial cells are clearly seen. Bars (d–f), 1 μm.
TEM showed STEC 784/1 inside vacuoles of variable size, some carrying two or more bacteria. The vacuolar membrane was well preserved and at the contact points with bacteria, no signs of intimate adherence were found (Fig. 2d). Bacteria appeared undamaged and seemed to be dividing (Fig. S3). SEM of STEC 784/1 and 282/2 showed bacteria being internalized, tightly wrapped in membrane folds. No cell membrane ruffling or blebbing was detected at the contact points with Fig. 2f). Cell entry of pathogens that induce the trigger entry mechanism such as Salmonella result in macropinosome formation, noticeable by the capture of extracellular fluid at the entry points. A fluid phase fluorescent marker (dextran-FITC) was used to look for macropinocytosis induction during STEC internalization at the peripheral cells from Caco-2 islets, with negative results (data not shown). As cellular actin cytoskeleton rearrangement enables the bacterial entry zipper and trigger mechanisms to be differentiated (Cossart & Sansonetti, 2004; Pizarro-Cerdá & Cossart, 2006; Veiga et al., 2007), the organization of actin microfilaments was investigated during the early stages of bacterial entry (30 min) using actin and bacteria double labelling. STEC internalization showed a discrete local actin rearrangement around the attached bacteria (Fig. 3a), resembling the pattern found with Y. enterocolitica (data not shown) whereas S. Typhimurium internalization induced massive actin rearrangement at the invasion site (Fig. 3a). After 30 min of infection when most STEC were intracellularly located or in the process of cell entry on the edges of the peripheral Caco-2 cells, actin aggregation resembling A/E lesions was not detected around or beneath the attached LEE-positive STEC bacteria, suggesting that their internalization does not require expression of the LEE-encoded apparatus (Fig. S4).

The higher STEC invasion level found in undifferentiated Caco-2 cells suggests that bacterial invasion proceeds through the basolateral side of enterocytes. Calcium chelators, such as EGTA, were found to reduce the extracellular calcium concentration and disrupted the cell–cell contacts in Caco-2 differentiated monolayers, rendering the basolateral domain accessible to STEC. STEC invasion was greatly enhanced (14- to 16-fold) by pre-treatment of fully differentiated Caco-2 monolayers with EGTA (Fig. 3b). As expected, basolateral invasion by Shigella was also enhanced (25-fold), whereas Salmonella apical invasion was only marginally increased. When the EGTA effect on early STEC invasion (15 min) was assessed by fluorescence microscopy (Fig. 3c), a great number of bacteria could be seen at the edges of the cells, mostly on the exposed basolateral region. On the other hand untreated monolayers had β1 integrin, a basolateral marker in Caco-2 cells (Beaulieu, 1992) restricted to the cell–cell contact areas and no bacteria could be detected at these sites.

**STEC survival in the intracellular compartment of Caco-2 cells**

It was established that in all the strains studied STEC persisted in the intracellular compartment of Caco-2 cells for up to 96 h of infection (Fig. 4a) without inducing alteration of cell viability as measured by the MTT assay (data not shown). In contrast, the intracellular multiplication of S. Typhimurium resulted in cell death after 48 h. A drop in the intracellular bacterial counts was noticed at 48 h or 72 h after infection, followed by an increase in the intracellular populations during the next 24 h.

The intracellular STEC viability was determined by the BacLight Live/Dead system. The invasion test was performed as described and the viable and nonviable intracellular bacterial population percentages at each infection time point are shown in Fig. 4(b). There was a significant increase (>50 %) in the nonviable subpopulation after 48 h of infection, followed by an increase in the viable subpopulation, keeping the death rates constant up to 96 h of infection. Such behaviour contrasts with that observed for S. Typhimurium, which had consistently low bacterial death rates (23 and 25 %) and a steady viable fraction increase of the bacterial population, suggesting sustained bacterial multiplication.

**DISCUSSION**

It is widely recognized that the bacterial adherence to enterocytes or other intestinal cells is the first step to colonization and eventual invasion by enteric pathogens. The cell-adherence ability also allows efficient toxin liberation on the cell surface or its introduction inside the target cells (Cossart & Sansonetti, 2004; Pizarro-Cerdá & Cossart, 2006).

Most of the examined STEC strains were able to adhere efficiently to the enterocytic cell lines and the adhesive efficiency differences did not seem to be associated with the carriage of LEE genes or genes of putative STEC adhesins. In most cases, adherence to the non-enterocytic cells was significantly lower than to the enterocytic cells. These findings suggest that in vitro studies designed to evaluate the role of candidate adhesins in STEC adherence should preferentially use enterocytic cell lineages. Comparison between adherence and invasion indexes showed that the adhesive ability does not translate directly into invasive efficiency and points towards a specific nature for the invasive process. This seems to depend more on specialized receptor molecules, distinct from those responsible for the adherence. These invasion-related receptor molecules were linked to the enterocytic cell origin and, at least for Caco-2 cells, were more accessible to STEC in non-polarized, undifferentiated cells.

Previously, McKee & O’Brien (1995) had attributed the invasion of STEC to the phagocytic activity of the enterocytic cell line they had used (HCT-8). In such cases,
it would be reasonable to expect a correlation between bacterial association with the host cell membrane and bacterial uptake, which did not happen to the tested STEC. Recently, Rogers et al. (2012) reported that expression of flagellin facilitates the invasion of HCT-8 cells by the LEE-negative STEC O113:H21 and found evidence that flagellin interacts with lipid rafts mediating signalling pathways involved in bacterial internalization. In this study, the aflagellated LEE-negative strain 208/2 showed an

**Fig. 3.** STEC invasion proceeds through the basolateral pole of Caco-2 cells by a zipper-like mechanism. (a) Double fluorescence labelling of cytoskeletal actin (green) and bacteria (red) infecting (30 min) undifferentiated, non-polarized Caco-2 cells. STEC 282/2 (O8:H19) shows discrete actin accumulation beneath bacterial cells whereas S. Typhimurium C20 induces intense actin mobilization at the site of bacterial entry (arrow). Bar, 5 μm. (b) STEC invasion (2 h) of differentiated Caco-2 monolayers after disruption of intercellular junctions by EGTA (1.0 mM, 1 h). Bacterial entry was assessed by the amikacin-killing assay. Sh. flexneri and S. Typhimurium were used as controls. Experiment was run in duplicate and repeated three times. Bars, mean ± SD; significant differences, *P<0.05. (c) Differentiated Caco-2 cell monolayers treated or not with EGTA and infected (15 min) with STEC 282/2. β1-integrin was labelled (green) as a marker of the basolateral cell domain. Bacteria (red) can be seen at the periphery of the EGTA-treated cells. DAPI was used to stain cell nuclei (blue). Non-EGTA-treated monolayers show β1-integrin localization on basolateral surfaces facing adjoining cells. Bar, 20 μm.

**Fig. 4.** Persistence of STEC within Caco-2 cells. (a) Survival of STEC within Caco-2 cells at 6, 24, 48, 72 and 96 h post-infection. Cells were infected with 10⁶ bacteria and after killing of extracellular bacteria, a low concentration of amikacin (10 μg ml⁻¹) was added to control extracellular replication of bacteria over the 96 h period. Caco-2 monolayers infected with S. Typhimurium survived for up to 48 h. Experiments were carried out in triplicate and repeated three times. Bars, mean ± SD. (b) Live and dead intracellular bacterial cell populations were assessed by the BacLight Live/Dead assay at 6, 24, 48, 72 and 96 h post-infection. Differential uptake of fluorescent marker stains live bacteria green and dead bacteria red. Infection was carried out as described in (a). y-Axis: percentages of live and dead bacteria in the intracellular population. Caco-2 monolayers infected with S. Typhimurium survived up to 48 h. Experiments were performed in triplicate and repeated three times. Significant differences, *P<0.05.
almost complete uncoupling between adherence and invasion to all the enterocytic cell lines tested. In spite of the efficient 208/2 adherence, cell invasion for that strain was virtually inexistent. Thus, it is possible that the non-invasive STEC strain 208/2 has an impaired invasion due to the absence of flagella. On the other hand, the LEE-positive STEC strain 2781/8 (O111:NM), although also devoid of flagellin, exhibited a marked invasive ability to T-84 cells.

Significant differences were found among the tested STEC strain invasion indexes. This is not unexpected given the high heterogeneity of the STEC group. This included strains of distinct serotypes, profiles of virulence genes and the ability to colonize the zoonotic reservoir and produce disease. In contrast with the other O157:H7 strains tested, the prototype strain EDL933, although efficient in adherence to the enterocytic cell lines, showed a reduced invasion. The reason for that behaviour is unknown, suggesting the existence of different invasive mechanisms or the participation of cellular and microbial factors that modulate the invasiveness of different STEC strains to different cellular lineages. Considering serotype O157:H7, several findings support the idea of inter-strain virulence variation. Molecular subtyping techniques (Kim et al., 1999; Yang et al., 2004; Manning et al., 2008) could identify subpopulations common to clinical isolates, and O157 strains appear to vary in their capacity to cause human infection and disease (McNally et al., 2001; Manning et al., 2008) or colonize zoonotic reservoirs (Kim et al., 1999; Lowe et al., 2009; Sharma et al., 2009).

For two LEE-negative and two LEE-positive STEC strains, the invasive activity was found to depend on the function of actin microfilaments and the phosphorylation of cell proteins by protein kinases, modulators of the actin polymerization-depolymerization processes. The general inhibition of protein kinases by staurosporine caused a reduction in STEC invasion, but except for strain EDL931 (O157:H7), tyrosine-protein kinases did not seem to be involved. These results indicate that cell entry by STEC requires a specific interaction between the invasive bacteria and the Caco-2 cells. Host cell cytoskeletal rearrangement and phosphorylation-mediated signal transduction are also involved in cell invasion by several pathogens (Cossart & Sansonetti, 2004). Our finding that colchicine treatment of undifferentiated Caco-2 cells results in a decreased invasion for LEE-positive STEC, but not for LEE-negative strains, suggests involvement of the microtubule network in the entry process for LEE-positive strains. Donnenberg et al. (1990) showed that microtubule inhibitors block invasion of EPEC, and it was recently found that microtubule disruption by EPEC and LEE-positive STEC is dependent on a functional type III secretion system (T3SS) and a translocated effector protein family, EspG (Clements et al., 2011). It was shown that microtubule disruption could contribute to the reduced invasion of the LEE-positive STEC tested. Concerning genistein effects, we found that only STEC O157:H7 invasion of undifferentiated Caco-2 cells was significantly reduced by the drug treatment.

Previous work with STEC O113:H21 showed conflicting results, as the genistein treatment of enterocytic HCT-8 cells, but not CHO-K1 cells, significantly decreased bacterial invasion (Luck et al., 2005; Rogers et al., 2012).

Human cell lineages, such as Caco-2 and T-84, behave phenotypically and functionally as enterocytes. Post-confluent monolayers display the property of expressing typical enterocytic differentiation due to the formation of tight junctions and segregation of the plasma membrane apical and basolateral domains. These changes mimic the differentiation process of intestinal epithelial cells during the crypt-to-villus migration. On the other hand, subconfluent cultures are mostly formed by non-polarized and undifferentiated cells. It was shown that enterocyte differentiation regulates the accessibility of cell receptors for bacterial adhesins and invasins (Rosa et al., 2001; Yamamoto et al., 2009).

For most STEC, a higher invasive efficiency to subconfluent, non-polarized Caco-2 cells was demonstrated, suggesting that internalization should preferentially occur at the basolateral pole. Giemsa-stained Caco-2 cell islets showed that the bacteria mainly associated with the outer edge of the non-polarized cells that surrounded the islets, and phase microscopy showed vacuole formation around the bacterial cells, suggesting their internalization. Even using higher inoculum (10⁸ c.f.u.) or longer infection times (6 h), such characteristics were not seen in the subconfluent or fully confluent monolayers of non-enterocytic cell lines. Further evidence was gathered showing that STEC gain access to the intracellular compartment through the basolateral pole of Caco-2 cells from differentiated monolayers treated with EGTA, a procedure that disrupts tight junctions and exposes receptors on the basolateral region (Rosa et al., 2001; Yamamoto et al., 2009).

In a previous study, Luck et al. (2005) showed that LEE-negative STEC, mostly of serotype O113:H21, were capable of invading CHO-K1 cells and remained within a membrane-bound vacuole. In this study, ultrathin sections of undifferentiated Caco-2 cells viewed in TEM showed large vacuoles, frequently containing more than one bacterium in the interior. However, although the Caco-2 cell basolateral pole seemed to be the preferential site for STEC invasion, these micro-organisms can also invade confluent, polarized monolayers of Caco-2 and T-84 cells, the basolateral poles of which are conceivably less accessible for bacterial interaction.

Bacterial invasion of non-phagocytic eukaryotic cells can proceed by direct engagement of cell surface receptors (zippering) or by direct translocation of bacterial proteins into the cytosol that promotes massive actin cytoskeleton recruitment, membrane ruffling and bacterial internalization within a macropinocytic vacuole (Cossart & Sansonetti, 2004; Veiga et al., 2007). Luck et al. (2005) suggested that STEC O113:H21 entry into CHO-K1 cells could proceed through a zipper-like mechanism. In this work, evidence was obtained suggesting that STEC invasion

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of undifferentiated Caco-2 cells was proceeded by zippering. Double immunofluorescence showed local actin accumulation and discrete membrane rearrangement around individual and small clusters of bacteria whereas a fluid-phase marker gave negative results for macropinocytosis at the sites of bacterial invasion. Analysis of STEC interaction with Caco-2 cells using SEM showed discrete membrane alterations, consistent with the zipper entry process.

To evaluate the kinetics of the invasion process and the intracellular fate of the internalized bacteria, a subset of STEC was chosen from serotypes O157:H7 (EDL 931), O103:H21 (0651/1) and O113:H21 (784/1), well known for their prevalence in the zoonotic reservoir and pathogenic potential (Caprioli et al., 2005; Beutin et al., 2005), and O8:H19 (282/2), a serotype recently isolated from cattle in Argentina and also found in human disease (Meichtri et al., 2004). Both the amikacin-killing assay and the double immunofluorescence microscopy showed the presence of intracellular bacteria after 5 min of infection of Caco-2 cells. A similar result was described by Luck et al. (2005) and these findings show that STEC are active invaders, gaining access to the intracellular compartment as fast as well-known invasive pathogens such as Salmonella or Yersinia. At least until the third hour of infection there was a steady increase in the number of intracellular bacteria. That increase did not seem to result from the sole internalization of bacteria adhered to the cell membranes, but mainly from the active multiplication of the bacterial population located in the intracellular compartment. All tested STEC strains persisted inside the cells for up to 96 h, with little or no damage to the cells whereas S. Typhimurium destroyed the cell monolayers after 48 h. These findings indicate that on achieving the intracellular environment, STEC do not behave as typical invasive pathogens like Salmonella, whose intracellular activity culminates with the destruction of the epithelial cell. On the contrary, they seem to follow an alternative pathway, attaining some degree of coexistence with the infected cell and surviving in the intracellular milieu for a long time. That strategy seems appropriate to assure the persistence of the micro-organism in the animal reservoir, as well as to sustain its continuous shedding to the environment.

Buchmeier & Libby (1997) used the BacLight Live/Dead vital staining to evaluate the amount of dead cells of S. Typhimurium inside macrophages. We found that antibacterial mechanisms operating inside enterocytes were clearly insufficient to control S. Typhimurium multiplication, allowing bacterial growth and a reduction in the bacterial death rates. After 48 h, the process culminated with the destruction of the cellular monolayer. For STEC, no bacterial growth curves were observed resembling those with Salmonella. After 48 or 72 h of intracellular residence, there was a reduction in the number of viable bacteria, suggesting the predominance of antibacterial mechanisms. Above 72 h the bacterial death rates remained constant, suggesting that intracellular persistence of STEC results from a balance between bacterial replication and death.

STEC carriage by healthy cattle can result in a persistent shedding of bacteria to the environment (Grauke et al., 2002) and the terminal rectal mucosa has been identified as the major site of O157:H7 colonization in cattle (Naylor et al., 2003; Low et al., 2005; Nart et al., 2008). Histopathological changes were observed in the colonization sites, including desquamation of enterocytes and neutrophil infiltration of the intestinal epithelia (Nart et al., 2008). Although LEE-encoded effector proteins might play a role in gut colonization (Naylor et al., 2005; Vlisidou et al., 2006; Dziva et al., 2007), LEE-negative STEC strains can colonize cattle efficiently, showing that other factors influence the persistence of STEC in the bovine intestine. Shen et al. (2011) working with rectal biopsies and with primary bovine rectal epithelial cell (PBRE) cultures, showed that a subpopulation of STEC O157:H7 was internalized, and that deletion of the tir gene, although impairing the intimate attachment and production of A/E lesions, did not affect bacterial internalization of the strain EDL933. Electron micrographs taken from infected PBRE cells showed microvilli-like membrane extensions surrounding the internalizing bacteria. These findings are consistent with this study's results that STEC strains derived from different sources can invade and replicate intracellularly within epithelial cells of enterocytic origin.

We found that, for most STEC, a significant proportion of enterocyte-associated bacteria gain access to the intracellular compartment via the basolateral pole. In the intestine, enterocyte differentiation occurs as epithelial cells migrate from the crypt up to the top of the villi and, after 2 to 5 days, the cells are desquamated into the intestinal lumen (McCracken & Lorenz, 2001). It is conceivable that during the differentiation process, incomplete segregation of basolateral molecules or enterocyte exfoliation can offer transitory opportunities for bacterial interaction. Besides that, several physiological and pathological factors are known to disrupt the epithelial barrier, such as neutrophil migration across intestinal cell monolayers and epithelial trauma, both leading to the exposition of basolaterally restricted proteins (Perdomo et al., 1994; McCormick et al., 1997). During healing of injured mucosa, the first response is the induction of epithelial migration, a process termed restitution (Basson et al., 1998). Cells involved in this process decrease or rearrange the expression of many differentiation characteristics, especially basolateral molecules involved in the cell–cell and cell–matrix interactions. Thus, cells engaged in the restitution process are potential targets for bacterial paracellular penetration, interaction with cell basolateral receptors, and entry. On the other hand, several enteropathogens may gain access to basolateral receptors and promote enterocyte invasion in vivo by transcytosis through M cells (Cossart & Sansonetti, 2004). Fitzhenry et al. (2003) and Chong et al. (2007) using an ex vivo model with human intestinal biopsies, showed that LEE-positive STEC strains from the serotypes O157:H7, O26:H11 and O103:H2 can induce A/E lesions mainly on the follicle-associated epithelium from the

F. Cordeiro and others
Peyer’s patch region of the distal ileum. Although the authors did not describe invasive activity or bacterial association with M cells, this finding leaves open the possibility that STEC can use this route to access the basolateral side of enterocytes.

Cell entry and intracellular survival may be an important virulence strategy for STEC, protecting the bacteria from host defence mechanisms operative on mucosal surfaces. This study’s results from long-term internalization and intracellular bacterial cell viability assays suggest that STEC strains assure their intracellular survival and persistence with reduced harm for enterocyte viability. It seems reasonable to speculate that survival of micro-organisms within enterocytes may provide the means for a persistent colonization of the zoonotic reservoir and assure efficiency of environmental and food transmission.

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**REFERENCES**


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