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ENTEROPATHOGENIC AND ENTEROHEMORRHAGIC *E. COLI*: ECOLOGY, PATHOGENESIS AND EVOLUTION

Topic Editors
Elizabeth L. Hartland and John M. Leong

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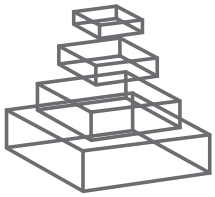

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ENTEROPATHOGENIC AND ENTEROHEMORRHAGIC *E. COLI*: ECOLOGY, PATHOGENESIS AND EVOLUTION

Topic Editors:
Elizabeth L. Hartland, The University of Melbourne, Australia
John M. Leong, Tufts University School of Medicine**,** USA

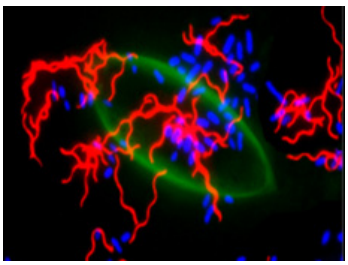

Image by Jorge A. Girón, taken from Saldaña Z, Sánchez E, Xicohtencatl-Cortes J, Puente JL and Girón JA (2011) Surface structures involved in plant stomata and leaf colonization by Shiga-toxigenic *Escherichia coli* O157:H7. *Front. Microbio.* 2:119. doi: 10.3389/fmicb.2011.00119.

Enteropathogenic and enterohemorrhagic *E. coli* are important causes of gastrointestinal disease worldwide. As part of their pathogenesis, EPEC and EHEC cause a distinctive lesion on the intestinal mucosa known as an attaching and effacing (A/E) lesion. A/E lesion formation requires a type III secretion system that injects multiple effector proteins into the cell. Despite their shared mechanism of intestinal colonization, EPEC and EHEC exhibit substantial differences in epidemiology and clinical disease. In particular, EHEC produces a potent Shiga toxin that is associated with development of the haemolytic uremic syndrome (HUS), an acute form of renal failure. This Research Topic will examine interactions between attaching and effacing bacteria and the host cell, and discuss EPEC/EPEC ecology, genomics and animal models of disease. Articles will centre on pathogen evolution, novel adhesins, type III effector biology and bacterium-host responses during infection.

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EDITORIAL
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Enteropathogenic and enterohemorrhagic *E. coli*: ecology, pathogenesis, and evolution

Elizabeth L. Hartland*[1,2] ***and John M. Leong*[3]***

[1] *Department of Microbiology and Immunology, University of Melbourne, Parkville, VIC, Australia*
[2] *Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, VIC, Australia*
[3] *Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA*
**Correspondence: hartland@unimelb.edu.au*

Edited by:
Yousef A. Kwaik, University of Louisville School of Medicine, USA

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Yousef A. Kwaik, University of Louisville School of Medicine, USA

The gastrointestinal pathogens enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC) continue to pose a threat to human health worldwide. While EPEC remains a significant cause of diarrhea in low-income countries, EHEC is more common as a food or water-borne pathogen in industrialized countries. A major difference between EPEC and EHEC is that EPEC has only a human reservoir of infection while EHEC is a zoonotic disease. Strains of EHEC are commensal in many ruminants, particularly cattle, and hence entry into the food chain through fecal contamination of food or water is a risk factor for infection. Another characteristic of EHEC but not EPEC is the production of Shiga toxins, which are associated with the development of severe complications of infection, namely hemorrhagic colitis (HC) and the hemolytic uremic syndrome (HUS). Whereas, HUS can affect patients of any age, EPEC remains a pathogen of infants less than 2 years of age (Robins-Browne and Hartland, 2002).

Despite their differing epidemiology and clinical manifestations, EPEC and EHEC are highly related and share many virulence determinants and features. Both pathogens share a distinctive mechanism of intestinal colonization known as attaching and effacing A/E lesion formation. A/E lesions are characterized by tight attachment of the bacteria to the enterocyte surface, the localized destruction of microvilli and massive ultrastructural changes underneath the adherent bacteria resulting from the accumulation of filamentous actin (Wong et al., 2011). The locus of enterocyte effacement (LEE) is essential for A/E lesion formation, and encodes a type III section system (T3SS) that translocates multiple effector proteins into the infected enterocyte. The LEE is quintessential for the definition of EPEC, as all strains of EPEC carry LEE and induce A/E lesions. The LEE is also used to define some Shiga-toxin producing *E. coli* (STEC) as EHEC such as O157:H7, although it role in defining EHEC is blurred somewhat by the existence of STEC that lack LEE yet cause HC and HUS and, as in the case of the recent EHEC O104:H4 outbreak, may be highly virulent (Frank et al., 2011). For true A/E pathogens, the LEE is absolutely required for infection and in EHEC O157:H7 its expression appears to be enhanced by passage in the mammalian gut (Brady et al., 2011), suggesting that animal-to-person or person-to-person transmission may be under positive selection during acute infection.

The LEE T3SS system is similar to those found in other pathogens but has an additional filament to the T3SS needle made of polymerized EspA subunits. EspA is essential for translocation to occur and may also provide some adhesive function during bacterial colonization. The most widely accepted model is that the hydrophobic LEE-encoded proteins, EspB and EspD, form a pore in the host cell membrane that provide a conduit for effector translocation through the T3SS. Together EspA, EspB, and EspD constitute the LEE translocon. A recent proposal is that type III secretion is a two-step process whereby surface-localized effector proteins are part of an intermediate effector-translocator complex that precedes effector translocation (Pilar and Coombes, 2011). Future work will be required to reconcile this model with the presence of the EspA filament that structural evidence suggests directly attaches to the T3SS needle (Sekiya et al., 2001).

Among the many translocated effector proteins, Tir plays a critical role in intimate attachment through binding the outer membrane protein intimin. Although intimin and Tir alleles are largely interchangeable despite sequence variation among different A/E pathogens, intimin has been associated with additional Tir-independent adhesive functions. This has also been explored as a source of EPEC/EHEC tissue tropism in the past (Fitzhenry et al., 2002). Here a Tir-independent role for intimin in the colonization of streptomycin-treated mice provides more evidence that in some experimental systems, intimin promotes intestinal colonization independently of its role in Tir-binding (Mallick et al., 2012).

While the intimin-Tir interaction has been intensely studied, not all effector proteins contribute to A/E lesion formation and cytoskeletal changes. It has recently emerged that several effector proteins play a role in dampening the inflammatory response. The effector proteins involved are all encoded outside the LEE and have novel enzymatic functions that target NF-κB and MAPK signaling. For example, NleE is a cysteine methyl transferase that methylates the zinc finger domain of TAB2/3, thereby inhibiting TAB2/3 interaction with ubiquitinated TRAF and blocking the phosphorylation of IκB by the IKK complex and hence the degradation of IκB (Newton et al., 2010; Zhang et al., 2011). NleC and NleD are zinc metalloproteases that cleave the p65 subunit of NF-κB and the MAPKs p38 and JNK, respectively (Baruch et al., 2011; Pearson et al., 2011). NleH has both

anti-apoptotic and anti-inflammatory effects acting in part by binding ribosomal protein S3 (Hemrajani et al., 2010; Gao and Hardwidge, 2011).

Although much research is aimed at understanding the function of the LEE and the translocated effector proteins [recently reviewed in Wong et al. (2011)], the carriage and transfer of virulence genes among different *E. coli* pathogens makes the study of other potential virulence factors critically important. For example, EhaJ is an autotransporter protein that is shared by strains of belonging to both EPEC and EHEC lineages. Autotransporters are highly prevalent in EPEC/EHEC genomes, and many contribute to inter-bacterial interactions and biofilm formation (Wells et al., 2010). EhaJ also has biofilm-producing properties when expressed in laboratory strains of *E. coli*, and this requires the function of a putative glycosyltransferase encoded by the adjacent gene, *egtA*. In addition, EhaJ has extracellular matrix binding properties, suggesting that the autotransporter may contribute to host infection. Other factors apart from the T3SS may also contribute to the ability of EHEC O157:H7 to persist and colonize the surface of plants, including pili and flagella (Saldana et al., 2011), as occurred in a large outbreak of EHEC O157:H7 that was spread by contaminated spinach (Wendel et al., 2009).

The reservoir of EHEC in cattle deserves particular attention as many LEE-independent factors, such as autotransporters, may contribute to persistence in this natural host. Indeed recent work reported here on the interactions of EHEC O157:H7 with bovine rectal epithelial cells suggests that the bacteria are internalized though an intimin- and Tir-independent mechanism. The rectum is the main site of EHEC O157:H7 colonization in cattle and acts as an ongoing source of contamination through fecal shedding (Low et al., 2005). To identify factors that contribute to the ability of EHEC to attach to rectal epithelial cells, here Bai et al. screened BAC clones derived from EHEC O157:H7 in a competition-based assay. They identified fimbriae and the autotransporter EhaA as candidates for EHEC-bovine epithelium interactions (Bai et al., 2011).

Because of its prominence in large outbreaks of disease, much research has focused on EHEC O157:H7 virulence. Nevertheless non-O157 EHEC, of which there are more than 100 serotypes, also have the capacity to cause HUS and we know far less about their genome structure and evolution (Coombes et al., 2011). As this information accumulates, we will have a much greater understanding of the relationship between EHEC/EPEC and other *E. coli* pathogens that all contribute to a common gene pool from which new pathogens, such as EHEC O104:H4, may emerge. Ongoing surveillance with high throughput genomics and molecular and infection studies are the keys to understanding the ecology and pathogenesis of EPEC and EHEC and are our only weapons against a fast evolving and dynamic group of pathogens.

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Internalization of *Escherichia coli* O157:H7 by bovine rectal epithelial cells

Haiqing Sheng[1], Jing Wang[1], Ji Youn Lim[1,2], Christine Davitt[3], Scott A. Minnich[1] and Carolyn J. Hovde[1]*

[1] *School of Food Science, University of Idaho, Moscow, ID, USA*
[2] *Department of Chemistry, Stanford University, Stanford, CA, USA*
[3] *Franceschi Microscopy and Imaging Center, Washington State University, Pullman, WA, USA*

Edited by:
Elizabeth L. Hartland, The University of Melbourne, Australia

Reviewed by:
Philip R. Hardwidge, University of Kansas Medical Center, USA
Jorge Giron, University of Florida, USA

***Correspondence:**
Carolyn J. Hovde, IDeA Networks of Biomedical Research Excellence, University of Idaho, Moscow, ID 83844-4207, USA.
e-mail: cbohach@uidaho.edu

Escherichia coli O157:H7 (O157) causes human diarrheal disease and healthy cattle are its primary reservoir. O157 colonize the bovine epithelial mucosa at the recto-anal junction (RAJ). Previous studies show that O157 at this site are not eliminated by aggressive interventions including applications of O157-specific lytic bacteriophages and other bactericidal agents. We hypothesize that some O157 at the RAJ mucosa are protected from these killing agents by host cell internalization. To test this hypothesis, rectal biopsies from O157 culture positive and negative cattle were analyzed by fluorescent microscopy and subjected to gentamicin protection assays. GFP-labeled bacteria were found located deep within the tissue crypts and a small number of O157 were recovered from rectal biopsies after gentamicin treatment. Primary bovine rectal epithelial (PBRE) cell cultures were incubated with O157 and subjected to gentamicin protection assays. Strains ATCC 43895, 43894, Sakai, and WSU180 entered the PBRE cells with different levels of efficiency ranging from 0.18 to 19.38% of the inocula. Intracellular bacteria were confirmed to be within membrane-bounded vacuoles by electron microscopy. Cytochalasin D curtailed internalization of O157 indicating internalization was dependent on eukaryotic microfilament assembly. Strain ATCC 43895 exhibited the highest efficiency of internalization and survived for at least 24 h within PBRE cells. Deletion mutation of intimin or its receptor in ATCC 43895 did not reduce bacterial internalization. This strain produced more biofilm than the others tested. Retrospective analysis of cattle challenged with two O157 strains, showed ATCC 43895, the most efficient at host cell internalization, was most persistent.

Keywords: *E. coli* O157:H7, cattle, internalization, epithelial cells, EHEC, bovine

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) cause human disease ranging from self-limited watery diarrhea to the life-threatening condition of hemorrhagic colitis and its sequelae – the hemolytic uremic syndrome (HUS; Karmali et al., 1983; Paton and Paton, 1998). *E. coli* O157:H7 is the predominate EHEC serotype isolated from disease outbreaks in North America, the United Kingdom, and Japan (Griffin and Tauxe, 1991; Smith, 1998; Mead et al., 1999; Michino et al., 1999). Healthy cattle are the major reservoir for *E. coli* O157:H7, the most common source for human infections (Hancock et al., 1994; Zhao et al., 1995; Chapman et al., 2001), and carry the bacteria with no apparent disease symptoms (Blanco et al., 1996; Besser et al., 1997; Hancock et al., 1997). Previous studies demonstrate that the recto-anal junction (RAJ) mucosa is the primary site of *E. coli* O157:H7 colonization in cattle (Grauke et al., 2002; Naylor et al., 2003; Cobbold et al., 2007; Dean-Nystrom et al., 2008), and the carriage of *E. coli* O157:H7 at this site is correlated to high level fecal shedding (Low et al., 2005). Rectal administration of *E. coli* O157:H7 results in efficient bacterial colonization at the RAJ mucosa that is maintained for >1 month (Sheng et al., 2004, 2006b). Because the terminal rectal mucosa may be the ideal target for controlling *E. coli* O157:H7 in cattle, we and others have applied O157-specific lytic bacteriophages (Sheng et al., 2006a; Rivas et al., 2010) or chemical chlorhexidine (Naylor et al., 2007) directly to this location. However, when these treatments are effective they reduce the level of the *E. coli* O157:H7 carriage, but never completely eliminate *E. coli* O157:H7 from the rectal mucosa.

Enterohemorrhagic *E. coli* and enteropathogenic *E. coli* (EPEC) belong to the "attaching and effacing (A/E) pathogen" category because of their ability to induce A/E lesions on intestinal epithelial cells. The characteristics of the A/E lesions include the localized effacement of the brush border microvilli, intimate bacterial attachment to the host epithelium, and the formation of cytoskeleton-rich pedestal-like structures beneath the adherent bacteria (Moon et al., 1983). The A/E pathogens share a highly homologous mobile genetic element called the locus of enterocyte effacement (LEE) pathogenicity island that is responsible for A/E lesion formation (McDaniel and Kaper, 1997; Hueck, 1998; Elliott et al., 1999, 2000). The LEE encodes a type III secretion system (T3SS), secreted proteins, chaperone, and regulatory molecules, and an outer membrane adhesion protein called intimin (Perna et al., 1998; Elliott et al., 1999). The T3SS is responsible for translocating into infected cells both LEE- and non-LEE-encoded (Nle)-effectors, including translocated intimin receptor (Tir), EspA, EspB, and EspD (Kenny et al., 1997; Knutton et al., 1998; Kenny, 2001). Tir is inserted into the host cell plasma membrane with Tir–intimin interaction triggering

signaling events leading to pedestal formation (Rosenshine et al., 1992a; Kenny et al., 1997). There are a number of studies that show the LEE encoding effector proteins play a role in ruminant intestinal colonization *in vivo* (Dean-Nystrom et al., 1998; Cornick et al., 2002; Dziva et al., 2004; Naylor et al., 2005; Sheng et al., 2006b; Vlisidou et al., 2006).

Enteropathogenic *E. coli* is usually considered an extracellular organism but under *in vitro* conditions bacteria can be seen intracellularly and this phenotype is dependent on a functional T3SS (Francis et al., 1991; Rosenshine et al., 1992a; Jepson et al., 2003). The effecter EspT facilitates EPEC internalization into non-phagocytic cells in a process involving Rac1 and Wave2 (Bulgin et al., 2009). In fact, in addition to the generation of intimate adherence and the A/E lesions, EHEC strains, like EPEC, are capable of efficient entry into a variety of epithelial cell types (Dibb-Fuller et al., 2001; Martinez and Hultgren, 2002; Uhlich et al., 2002, 2009; Abu-Ali et al., 2010), though they are also regarded as extracellular pathogens. Internalization of EHEC O157:H7 by human epithelial cells is also known (Oelschlaeger et al., 1994) and Dibb-Fuller et al. (2001) demonstrate *E. coli* O157:H7 is internalized in several distinct bovine cell lines. The interaction of *E. coli* O157:H7 with bovine host cells and the mechanism(s) that lead to the specific tropism and persistence at the rectal mucosa are not fully understood. We speculated that the bovine epithelial cells at the terminal rectal mucosa may internalize a subpopulation of *E. coli* O157:H7 and these intracellular bacteria may contribute to the persistence of *E. coli* O157:H7 at this colonization site. The aims of this study were to (1) describe *E. coli* O157:H7 in biopsy tissue taken from *E. coli* O157:H7 culture positive cattle; and (2) investigate the internalization of *E. coli* O157:H7 using primary bovine rectal epithelial (PBRE) cells.

MATERIALS AND METHODS

BACTERIA AND CULTURE

All strains used in this study are listed in **Table 1**. The clinical isolates were chosen because they are well characterized human pathogens and two have full DNA sequence known (ATCC 43895, also referred to as EDL933, and Sakai). All have been used previously by our laboratory and others in cattle trails of *E. coli* O157:H7 carriage. *E. coli* O157:H7 and other *E. coli* strains were grown in Luria–Bertani (LB) broth or agar. Recombinant *E. coli* O157:H7 43894 GFPuv was grown in LB with 100 μg/ml ampicillin.

GENE MANIPULATION

To make a mutant deficient in expression of intimin or Tir in *E. coli* O157:H7 43894, the λ-Red recombinase system (Datsenko and Wanner, 2000) was used for *eae* or *tir* gene deletion, respectively. The plasmid pKD4 was used as a template to amplify a kanamycin resistance gene, and the same primers were used as described, previously (Sheng et al., 2006b).

CATTLE CHALLENGE

All animal procedures were approved by the Institutional Animal Care and Use and Biosafety Committees and were strictly followed in this study. Seven to 10-month-old Holstein steers were housed in a quarantined facility at the University of Idaho Agriculture Experiment Station as previous described (Sheng et al., 2006b). Steers were given a single rectal application of 10^7 CFU of *E. coli* O157:H7 strains 43895, 43894, or 43894 GFPuv as previously described (Sheng et al., 2004). Retrospective analysis of strain persistence used previously published and unpublished cattle rectal application challenge culture data collected by our laboratory since 2004 (Kudva et al., 2006; Sheng et al., 2006b; and unpublished data).

PREPARATION OF RECTAL BIOPSY TISSUE

Feces were manually removed from the cattle rectum and mucosal biopsies were taken from the rectal mucosa 10–15 cm proximal to the anus using a standard bone curette. The wet weights of biopsy tissues ranged from 30 to 60 mg. The biopsy tissues were rinsed with cold phosphate buffered saline (PBS) and held in cold Hanks' balanced salt solution (HBSS) on ice for transport to the laboratory.

TISSUE-EMBEDDED *E. coli* O157:H7 RECOVERY ASSAY

The terminal rectal tissue biopsies from *E. coli* O157:H7 culture positive cattle were transferred into sterile 10-cm Petri dishes with 15 ml PBS, fecal debris, and clotted blood was trimmed away and

Table 1 | Bacterial strains used in this study.

Strains	Description	Source or reference
43895 (EDL933)	*E. coli* O157:H7 ATCC 43895, a clinical isolate from ground beef, *stx1*$^+$/*stx2*$^+$	ATCC
43894	*E. coli* O157:H7 ATCC 43894, a clinical isolate from human stool, *stx1*$^+$/*stx2*$^+$	ATCC
Sakai	*E. coli* O157:H7, a clinical isolate, *stx1*$^+$/*stx2*$^+$	Lim et al. (2010), Tatsuno et al. (2001)
43894 GFPuv	43894 carry pCRII-GFPuv and produces strong fluorescence when excited by UV light. Amp^R	Laboratory stock
43895Δ*eae*	43895 with intimin gene deletion	Sheng et al. (2006b)
43895Δ*tir*	43895 with Tir gene deletion	Sheng et al. (2006b)
43894Δ*eae*	43894 with intimin gene deletion	This work
43894Δ*tir*	43894 with Tir gene deletion	This work
WSU180	*E. coli* O157:H7, a bovine isolate, *stx1*$^+$/*stx2*$^+$	Rice et al. (2003)
K-12	*E. coli* MG1655 strain	Laboratory stock

ATCC, American type culture collection, Manassas, VA, USA.

the remaining tissue was cut into fine pieces with sterile scissors. The minced tissue was recovered by centrifugation at 500×*g* for 5 min, the supernatant was removed, 100 μg/ml gentamicin in HBSS was added, and the tissue was incubated with shaking (150 rpm) at 37°C for 2 h to kill extracellular bacteria. The biopsy tissues were then washed three times with PBS, lysed with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS by vigorous vortexing. Serial dilutions of the mixture were plated on sorbitol MacConkey agar (SMAC) supplemented with cefixime (50 μg/l; Lederle Laboratories), potassium tellurite (2.5 mg/l; Sigma), vancomycin (40 mg/l; Sigma), and 4-methylumbelliferyl-β-D-glucuronide (0.1 μg/ml, Biosynth AG, Staad, Switzerland). The plates were incubated at 37°C overnight. *E. coli* O157:H7 colonies were identified and confirmed as O157 by latex agglutination as previous described (Sheng et al., 2008).

PREPARATION OF TISSUE BIOPSIES FOR MICROSCOPY

To prepare cryostat sections, biopsy tissues from the terminal rectum of 43894 GFPuv culture positive and negative control cattle were fixed with 4% (w/v) paraformaldehyde for 30 min, and infiltrated with up to 25% (w/v) sucrose in PBS (7.5, 15, 25% step gradient). Tissue was oriented to obtain optimal apical to basolateral cryostat cross sections (5–10 μm) that were placed on polylysine-coated slides, air dried, and observed using fluorescence microscopy.

PRIMARY BOVINE EPITHELIAL CELL ISOLATION AND CELL CULTURE

Primary bovine rectal epithelial cells were isolated from rectal biopsies as described by others with some modifications (Follmann et al., 2000; Dziva et al., 2007; Stamm et al., 2008). Briefly, rectal biopsies were taken from *E. coli* O157:H7 negative steers, cleaned, and cut as described above. The treated tissues were digested in Dulbecco's modified Eagle medium (DMEM) containing 1% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Invitrogen), 30 mg/ml streptomycin (Invitrogen), 25 mg/ml gentamicin (Invitrogen), 75 U/ml collagenase (Sigma), and 20 mg/ml accutase (Millipore, Billerica, MA, USA) with gentle shaking at 37°C until isolated crypts were observed under microscopy. A series of differential centrifugation steps with DMEM containing 2% (w/v) sorbitol was used to enrich the disassociated crypts. The crypt cell pellet was resuspended in cell culture medium [DMEM, 2.5% (v/v) FBS, 0.25 U/ml insulin (Sigma), 10 ng/ml epidermal growth factor (EGF; Sigma), 100 U/ml penicillin, 30 mg/ml streptomycin, and 30 mg/ml gentamicin]. Approximately 400 crypts are seeded per well into 24-well culture plates precoated with collagen (Becton Dickinson Labware, Bedford, MA, USA). Media was supplemented with 100 μg/ml *cis*-OH-proline (Sigma) to selectively eliminate fibroblasts from the cultures (Willis et al., 2005). Confluence of the cultured cells was reached within 6–7 days and contained approximately 1.8×10^5 cells/well. The characteristics of the epithelial cells were confirmed by immunocytochemistry as described previously (Hoey et al., 2003).

ADHERENCE AND INTERNALIZATION ASSAYS

The bacterial internalization by the PBRE cells was measured using a standard gentamicin protection assay (Small et al., 1987; Matthews et al., 1997). Monolayers of the PBRE cells were washed twice with HBSS and infected with 1 ml of the cell culture medium without antibiotics and FBS (internalization medium) at a MOI of 100 bacteria/epithelial cell. After 4 h incubation at 37°C with 5% CO_2, unattached extracellular bacteria were removed using a serological pipette and the infected monolayers were washed three times with HBSS. The total number of cell-associated bacteria (adhered and internalized) were measured by disrupting the monolayers for 10 min with 0.1% Triton X-100 in 0.1 M PBS (pH 7.2) and gentle vortexing. Serial dilutions were plated on LB agar to determine bacterial numbers. A standard Giemsa stain procedure was used in replicate sample wells to view the interaction of the bacteria with PBEC cells, microscopically. For measurement of internalization, fresh internalization medium containing 100 μg/ml of gentamicin was added to kill adhered extracellular bacteria. After incubation for an additional 2 h, the monolayers were washed three times with HBSS without Ca^{2+} or Mg^{2+} and epithelial cells were lysed by addition of 100 μl of 0.5% trypsin–EDTA (Sigma) and 900 μl of 0.05% Triton X-100 for 5 min. Samples were removed, serially diluted, and plated on LB agar to determine the number of CFU. Internalization levels were expressed as the number of CFU recovered/well or as a percentage of the original inoculum resisting gentamicin treatment.

INTERNALIZATION INHIBITION ASSAY

To assess the effect of eukaryotic cell inhibitors on bacterial internalization, the PBRE cell monolayers were incubated with cytochalasin D (1 μg/ml, Sigma), colchicine (1.25 μM, Sigma), compactin (25 μM, Sigma), or genistein (50 μM, Sigma) prior to addition of bacteria as described previously (Matthews et al., 1997). Cytochalasin D and colchicine were added to the monolayers 30 min prior to inoculation, genistein 15 min prior to inoculation, and compactin 18 h prior to inoculation. All of the inhibitors were present throughout the 4-h bacterial incubation period. Controls verified that inhibitors did not affect eukaryotic cell viability by trypan blue exclusion and bacterial viability by plate count. Data are expressed as means of the averages obtained from three experiments.

IMMUNOFLUORESCENCE MICROSCOPY

To visualize the location of *E. coli* O157:H7 in the biopsy tissue sections, 43894 GFPuv which expresses green fluorescent protein was applied at the rectal mucosa of two steers. The GFP carry-plasmid was stable in 43894 without antibiotic supplement. The cryostat sections were examined by epifluorescence microscopy using an Olympus BX51 microscope with a 100× oil immersion objective (Olympus, Tokyo, Japan). Digital images were acquired using an Olympus DP-70 digital camera and merged using DP manager software version 1.1.1.71.

ELECTRON MICROSCOPY

The PBRE cells were infected with 43894 or 43895 for 4 h and fixed in 2.5% glutaraldehyde 2% paraformaldehyde in 0.1 M cacodylate buffer, followed by post-fixation in 2% osmium tetroxide in 0.1 M in 0.1 M cacodylate buffer for 1 h. The cells were dehydrated in an ethanol series, followed twice by 100% acetone, infiltrated with Spurr's resin and polymerized overnight at 70°C. Thin (90 nm) sections were stained with 4% uranyl acetate and Reynolds lead before

viewing with a JEOL 1200 EX JEM transmission electron microscope located at the Franceschi Microscopy and Imaging Center (FMIC), Washington State University; Pullman, WA, USA.

BIOFILM FORMATION ASSAY

The biofilm formation assay was performed using a microtiter plate method as described previously (Djordjevic et al., 2002) with some modifications. Briefly, a single colony of each *E. coli* O157:H7 strain was inoculated into 4 ml LB broth and incubated at 37°C overnight with aeration. Then, 5 µl overnight culture was inoculated into 96 well microtiter plates (Corning, Inc., New York, NY, USA) containing 160 µl minimal salt medium (MSM) supplemented with 1 mg/l of yeast extract and 0.04% glucose and incubated at 37°C for 24 h under stationary conditions. Each strain was inoculated in triplicate. Following incubation the plates were washed vigorously with DI water and stained for 15 min with crystal violet (1%) and rinsed vigorously with DI water again to remove unattached cells and residual dye. Biofilm formation was evaluated by measuring the absorbance of solubilized dye in 95% ethanol at 595 nm using a PowerWave XS plate reader (Bio-Tek, Winooski, VT, USA). The biofilm formation experiments were performed in triplicate.

STATISTICAL ANALYSIS

Graphs were drawn using Microsoft Excel and GraphPad Prism software 4.0 (San Diego, CA, USA). The numbers of *E. coli* O157:H7 recovered from gentamicin protection assay were transformed to $\log_{10}$ values. The data were analyzed by GraphPad Prism software 4.0. The data were analyzed by the chi-square test unless the variables needed the Statistical Analysis's *t* test procedure. For retrospective analysis of strain persistence, survival analysis was used to compare the duration of *E. coli* O157:H7 infections of two groups of cattle rectally inoculated with 43895 or 43894. Survival time was defined as the number of days a rectal swab sample was continuously cultured positive for *E. coli* O157:H7. The Log-rank (Mantel–Cox) test was conducted to evaluate the statistical significance of the difference between durations of carriage caused by 43895 and 43994.

RESULTS

E. coli O157:H7 IN BIOPSY TISSUE SURVIVED GENTAMICIN TREATMENT AND WAS DEEP IN THE MUCOSAL CRYPTS

To determine if *E. coli* O157:H7 were internalized by bovine epithelial cells at the terminal rectum, rectal tissue biopsies from culture positive cattle were analyzed using a gentamicin protection assay. Two groups of cattle received rectal application of *E. coli* O157:H7 strain 43895 or 43894 at day 0, and rectal tissue was biopsied on days 1, 4, and 7 post-challenge. The average tissue sample was 47 mg and contained 12 crypts (data not shown). **Table 2** shows that the number of *E. coli* O157 recovered from rectal biopsy tissues after 2 h gentamicin treatment. On each sampling time (day 1, 4, and 7), higher numbers of the bacteria were recovered after gentamicin treatment from the rectal biopsies of the 43895-infected cattle compared to the 43894-infected cattle ($P < 0.05$). The data indicated that more 43895 were protected from gentamicin killing than 43894, and suggest the two strains were different in their interaction with bovine host cells.

Table 2 | *Escherichia coli* O157:H7 from bovine rectal tissue biopsy.

Strain	ATCC 43894[a]	ATCC 43895[a]
Days post-challenge	CFU[b]/crypt[c] (mean ± SD)	CFU[b]/crypt[c] (mean ± SD)
1	34 ± 15	278 ± 114*
4	241 ± 99	1788 ± 865*
7	30 ± 21	523 ± 102*

[a]Approximately 10^7 CFU E. coli O157:H7 ATCC 43894 or 43895 were inoculated at the terminal rectal mucosa in two separate groups of four steers on day 0.
[b]CFU recovered after gentamicin treatment.
[c]A minimum of three biopsies were taken from each animal on each sampling day and each biopsy contained an average of 12 crypts.
**Indicates a higher number of 43895 recovered compared to 43894 on the respective sampling days ($P < 0.05$).*

To localize *E. coli* O157:H7 in biopsy tissue, 43894 GFPuv was applied to the terminal rectal mucosa of steers and 4 days after challenge, biopsies of rectal tissue were taken and frozen sections prepared for fluorescence microscopy. Tissue sections of the first 10 µm from the lumen side showed green fluorescent bacteria adhered to the surface of the rectal mucosa (**Figure 1A**). Interestingly, 43894 GFPuv were visible in sections 30–40 µm proximal to the lumen surface, deep within the crypt (**Figure 1B**). No fluorescent bacteria were seen in samples from culture-negative cattle (**Figure 1C**). The cellular architecture and crypt bodies were visible in tissue sections from culture-negative steers due to weak tissue autofluorescence (**Figure 1C**) and in rectal tissue biopsies stained with H and E (**Figure 1D**).

PBRE CELL CULTURE WAS ESTABLISHED AND CHARACTERIZED

To test the internalization of *E. coli* O157:H7 by bovine rectal epithelial cells, PBRE cells were obtained from rectal tissue biopsies. After mechanical disruption and enzymatic digestion of the tissue, crypt bodies were obtained (**Figure 2A**). With appropriate culture conditions and cell densities, a confluent epithelial cell layer derived from the crypts was obtained after ~6 days (**Figure 2B**). The epithelial characteristics of the cultured cells were confirmed using pan-cytokeratin antibody specific for the intermediate filaments present in epithelial cells and anti-vimentin labeled V79 fibroblasts served as a negative control (data not shown).

E. coli O157:H7 ADHERED TO AND WERE INTERNALIZED BY THE PBRE CELLS

Cell association and the gentamicin protection assays were employed to quantitatively assess adherence and internalization of four different *E. coli* O157:H7 strains by PBRE cells. The bacterial strains included three *E. coli* O157:H7 isolates from outbreaks of human disease: 43895, 43894, Sakai, a bovine isolate:WSU180, and a non-pathogenic laboratory strain of *E. coli*: K-12 MG1655. All strains grew at indistinguishable rates both in LB and minimal medium. **Table 3** shows the numbers of cell-associated and internalized bacteria. The numbers of cell-associated and internalized bacteria varied significantly among the *E. coli* O157:H7 strains with 43895 values significantly higher compared with to 43894, Sakai, and WSU 180 ($P < 0.001$). Not only were high numbers of 43895 internalized by the

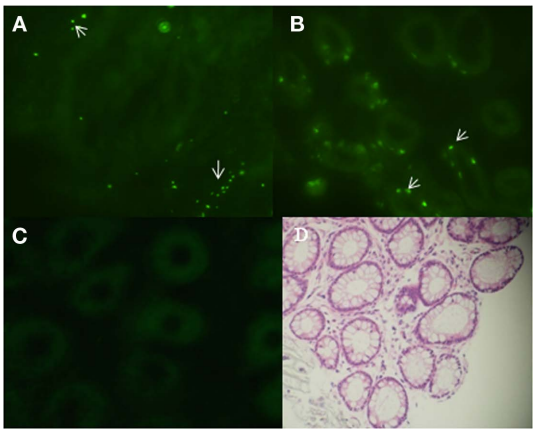

FIGURE 1 | *Escherichia coli* O157:H7 penetrated into the crypts of the bovine terminal rectal mucosa. Cattle were challenged with *E. coli* O157:H7 43894 GFPuv by standard rectal application of bacteria and negative control animals received no bacteria. Rectal mucosal biopsies were taken using a curette 4 days post-challenge. Tissue was processed, cryosectioned, and observed by light or fluorescent microscopy. Representative biopsy sections from O157 culture-negative and -positive cattle are shown. The sections were cut sequentially from the lumen side. **(A)** *E. coli* O157:H7 43894 GFPuv (arrow) visible 10 μm from the lumen surface and **(B)** around crypts at 30–40 μm from the lumen surface. **(C)** Biopsy tissue section from an O157 culture-negative animal. **(D)** Hematoxylin and eosin-stained section showing crypts of the terminal rectal mucosa. Crypts show autofluorescence with the fluorescence microscopy (original magnification ×200).

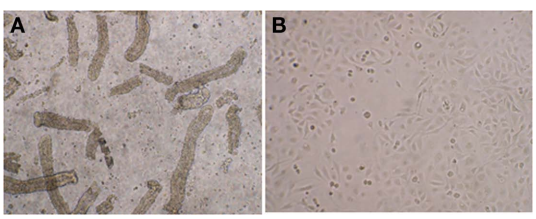

FIGURE 2 | Establishment of primary bovine rectal epithelial (PBRE) cells. **(A)** Crypt bodies released from biopsies of the terminal rectal mucosa after 2 h enzymatic digestion at 37°C. Incubation at 37°C in supplemented DMEM medium for 6 days gave rise to **(B)** a confluence monolayer of epithelial cells. Both representative images taken by inverted microscopy (original magnification, ×100).

PBRE cells, but a mean of 19.38% of the original number of 43895 survived after 2 h gentamicin treatment. This higher internalization level of 43895 in the PBEC cells compared to 43894 is in agreement with the biopsy data that showed higher numbers of 43895 than 43894 were recovered from the biopsy tissues after gentamicin treatment. Cell association and internalization of *E. coli* K-12 MG1655, used as a non-pathogenic *E. coli* control, was significantly lower than all tested *E. coli* O157:H7 strains ($P < 0.05$).

E. coli O157:H7 43895 WERE ASSOCIATED WITH PBRE CELLS IN AN AGGREGATIVE PATTERN AND INTERNALIZED BACTERIA WERE IN VACUOLES

Giemsa staining showed 43895 were associated with PBRE cells with numerous bacteria aggregated together. Other tested *E. coli* O157:H7 strains, 43894 (**Figure 3**), Sakai (data not shown), and WSU180 (data not shown) had fewer PBRE cell-associated bacteria and were mostly as single bacterial cells. PBRE cells were examined by TEM 4 h post-incubation with bacteria and intracellular *E. coli* O157:H7 were observed in membrane-bound vacuoles (**Figures 3B,C**). The PBRE cells frequently internalized ten to twenty 43895 (**Figure 3**) with >80% of PBEC cell monolayer having engulfed bacteria (data not shown), while only one to three 43894 cells were internalized (**Figure 3**) by approximately 15% of the PBEC cell monolayer (data not shown). These TEM results were consistence with the internalization assay results (**Table 3**) in which a 100-fold higher recovery of 43895, compared to 43894, occurred. It also appeared that 43895 may be dividing in the PBRE vacuoles (**Figure 3D**), although this was not tested further. These data confirmed that 43895 had an exceptional ability to be internalized by bovine host cells compared to other three tested *E. coli* O157:H7 strains.

E. coli O157:H7 43895 SURVIVED WITHIN THE PBRE CELLS

To investigate the survival of *E. coli* O157:H7 in the PBRE cells, the number of intracellular bacteria was determined at various times after gentamicin exposure and expressed as percentages of the total bacteria recovered. The number of 43895 or 43894 recovered after 6 h exposure to gentamicin decreased to 42.1 and 23.2% of the total bacteria recovered at 2 h, respectively (**Figure 4**). After 24 h exposure to gentamicin, the number of intracellular bacteria was dramatically decreased. The survival rate of 43895 was decreased to 25.9% while <1% of 43894 survived at 24 h (**Figure 4**). The number of PBRE cells attached to the wells and infected by 43895 decreased to 94% at 6 h and to 85% at 24 h of gentamicin treatment compared to the number at 2 h (data not shown). This decrease in 43895-infected cells suggested that some (6–15%) 43895-infected PBRE cells were destroyed over the course of the experiment and released bacteria that would be killed by gentamicin. Thus, the decreased number of 43895 recovered after 6 and 24 h exposure to gentamicin, may include these released cells. Almost all (>99%) attached PBRE cells incubated with 43895 or 43894 excluded the trypan blue dye. Similar and minimal tissue culture cellular debris/destruction was noted with 43894 or 43895 during the 4-h incubation period (data not shown). In addition, the culture media containing gentamicin was tested and remained sterile indicating that the bacteria recovered were intracellular.

CYTOCHALASIN D INHIBITED INTERNALIZATION OF *E. coli* O157:H7 BY THE PBRE CELLS

To identify host cell factors involved in internalization of 43895 and 43894, PBRE cells were treated with eukaryotic cell inhibitors known to block bacterial invasion (Rosenshine et al., 1992b; Matthews et al., 1997; Martinez and Hultgren, 2002). Treatment with cytochalasin D, an inhibitor of actin microfilaments, did not modify the number of cell-associated bacteria ($P > 0.05$; **Figure 5A**). In contrast, its addition significantly inhibited the entry of both 43895 and 43894. The numbers of intracellular bacteria decreased by 97.4 and 99.2% at 0.5 μg/ml ($P < 0.001$) and by 98.7 and 99.6% at 1 μg/ml concentration ($P < 0.001$), for 43895 and 43894, respectively (**Figure 5B**). Inhibitory assays showed that colchicines, a eukaryotic microtubule formation inhibitor, compactin, a pan-Rho GTPase inhibitor, and Genistein, a tyrosine kinase inhibitor did not

Table 3 | Internalization of *E. coli* O157:H7 and K-12 by primary bovine rectal epithelial cells ($n = 3$).

Strains	Cell-associated[a] (CFU × 10^7/well)	Internalized[b] (CFU × 10^4/well)	% Internalization[c]
ATCC 43895	11.88 ± 3.75*	547.12 ± 110.13*	19.38 ± 3.91*
ATCC 43894	2.09 ± 0.82	5.32 ± 2.29	0.20 ± 0.09
Sakai	2.45 ± 0.73	5.41 ± 2.21	0.19 ± 0.08
WSU180	2.23 ± 0.77	4.89 ± 3.05	0.18 ± 0.12
E. coli K-12	1.02 ± 0.39	1.40 ± 0.49*	0.07 ± 0.02*

[a]*CFU recovered from washed, lysed monolayers after 4 h incubation with bacteria.*
[b]*CFU recovered from lysed monolayers after 4 h incubation with bacteria and 2 h gentamicin treatment.*
[c]*Percentage of inoculae surviving gentamicin treatment.*
**Indicates a difference ($P > 0.05$) compared with the other E. coli O157:H7 strains.*

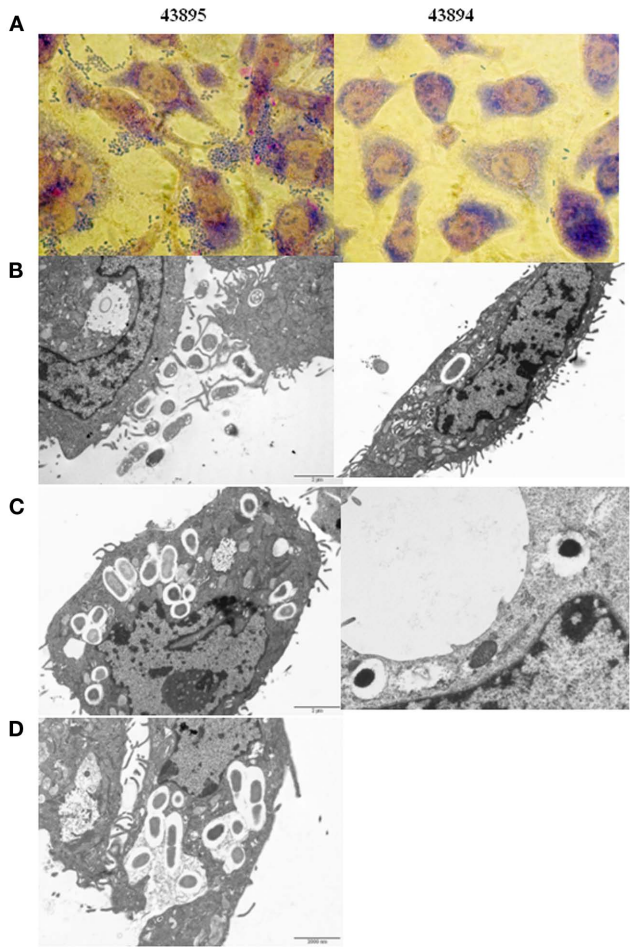

FIGURE 3 | *Escherichia coli* O15:H7 adheres to and enters the PBRE cells. *E. coli* O15:H7 43894 or 43895 were incubated with the monolayer of PBRE cells at 100 MOI for 4 h. More 43895 bacteria adhered to and were internalized by the PBRE cells compared to 43894. **(A)** Giemsa staining showing 43895 aggregative adherence pattern (inverted microscope, original magnification, ×1000). **(B,C)** Transmission electronic microscope showing extracellular and intracellular bacteria. **(D)** 43895 appeared to divide within the vacuoles of the PBRE cells (Original magnification, ×10000).

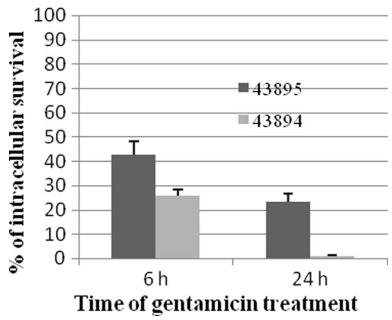

FIGURE 4 | Survival of *E. coli* O157:H7 in PBRE cells. Bacteria were incubated with monolayers of the PBRE cells at 100 MOI for 4 h. Gentamicin (100 μg/ml) was added for 2, 6, or 24 h incubation. Cells were lysed and plated to LB agar to enumerate surviving bacteria. The bacterial CFU recovered after 2 h Gentamicin treatment was set at 100%. The percentage of intracellular bacteria surviving after 6 and 24 h are shown and each value is the mean of three separate experiments.

influence PBRE cell internalization of 43895 or 43894 (data now shown). The data indicated that the internalization process of *E. coli* O157:H7 was dependent on actin microfilament assembly.

INTIMIN AND TIR ARE NOT REQUIRED FOR *E. coli* O157:H7 INTERNALIZATION

Our previous study shows that intimin and its receptor-Tir are essential for efficient *E. coli* O157:H7 colonization at the RAJ mucosa (Sheng et al., 2006b). To test whether intimin or Tir had a role in internalization by the PBRE cells, we examined the ability of intimin or Tir deletion mutants of both 43894 and 43895 to enter the PBRE cells. Although internalization was slightly reduced for 43894Δ*eae*, a mutant in which intimin was deleted, it was not significantly different from the wild-type. However, the slight reduction in PBRE cell internalization of the 43894Δ*tir* mutants (log value 4.03 ± 0.11) compared to the wild-type (log value 4.73 ± 0.17) was significant ($P < 0.05$; **Figure 6**), although not dramatic. Similar trends were observed in Tir and intimin mutants of 43895. Both 43895Δ*eae* and Δ*tir* showed reduced levels of internalization (log value 5.95 ± 0.25, 6.03 ± 0.21, respectively); however, these decreased levels were not significant compared to wild-type 43895 (log value 6.33 ± 0.19; $P > 0.05$). Notably, the levels of 43895Δ*eae* and Δ*tir* internalization were significantly higher than the wild-type 43894, its corresponding mutants (43894Δ*eae* and 43894Δ*tir*; $P < 0.001$; **Figure 6**). The data showed strain variation in that deletion of Tir did not affect the high internalization properties of 43895, but did reduce the lower internalization properties of strain 43894 in PBRE cells.

43895 IS MOST EFFICIENT IN BIOFILM FORMATION

Because the pattern of adherence appear aggregative for 43895, the *E. coli* O157:H7 strains used in this study were assessed for biofilm formation in a standard microtiter plate crystal violet biofilm assay in which formation of bacterial matrices attached to the plastic wells were quantified. 43895 formed more biofilm ($P < 0.05$), with mean optical densities of 0.55 ± 0.043, than 43894 (0.09 ± 0.02), Sakai (0.14 ± 0.05), or WSU180 (0.11 ± 0.03; **Figure 7**). The enhanced ability of 43895 biofilm formation correlated with its enhanced ability to adhere to and enter PBEC cells.

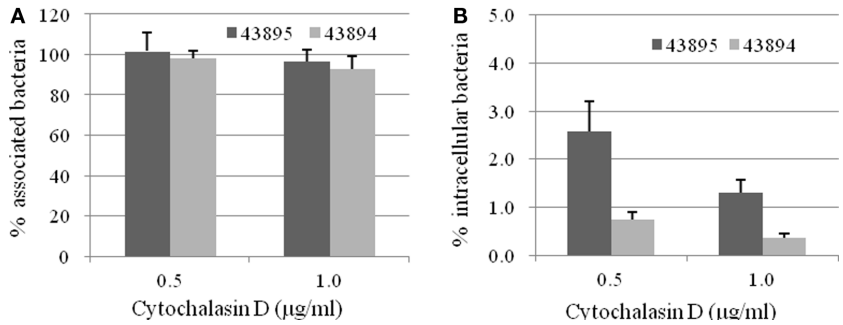

FIGURE 5 | Effect of cytochalasin D on *E. coli* O157:H7 association with and internalization by the PBRE cells. The PBRE cell monolayers were pretreated with cytochalasin D for 30 min before the addition of *E. coli* O157:H7 at 100 MOI and incubated for 4 h in the presence of the inhibitor. **(A)** Total cell-associated bacteria were quantified after PBRE cell-lysis with 0.1% (v/v) Triton X-100. **(B)** Intracellular bacteria were quantified after gentamicin treatment for 2 h. Results are expressed as cell-associated or intracellular bacteria relative to those obtained without inhibitor, taken as 100%. Each value is the mean of three separate experiments.

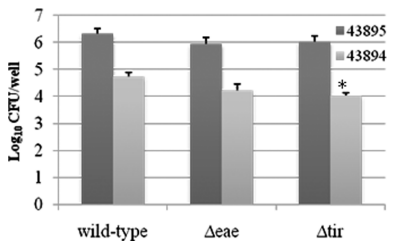

FIGURE 6 | The effect of the deletion of intimin or Tir on PBRE cell internalization of *E. coli* O157:H7. *E. coli* O157:H7 43894 or 43895 and the derivatives lacking intimin (Δ*eae*) or Tir (Δ*tir*) with the monolayer of the PBRE cells at 100 MOI. The number of bacteria recovered after gentamicin treatment indicated the number internalized by the epithelial cells. The experiment was repeated three times with three replicated wells. Bars represent means ± SE. Significant difference compared with wild-type *E. coli* O157:H7 is indicated by the asterisk ($P < 0.05$).

STRAIN 43895 PERSISTS LONGER THAN 43894 IN CATTLE

Retrospective analysis of previous studies in which cattle were challenged with a single rectal application of 10^7 CFU of *E. coli* O157:H7 43895 or 43894 was done to compare durations of bacterial carriage. The proportion of animals that were culture positive for *E. coli* O157:H7 from each group on each sampling day was compared and shown in **Figure 8**. There was a difference ($P < 0.05$) between 43895 challenged animals ($n = 22$) and 43894 challenged animals ($n = 14$) determined using the Log-rank (Mantel–Cox) test. The median duration of carriage with 43895 or 43894 was 34 and 25 days, respectively. The detection limit for *E. coli* O157:H7 carriage, as previously described for enrichment culture, was 10 CFU/swab (Kudva et al., 2006; Sheng et al., 2006b). This result indicated that *E. coli* O157:H7 strain 43895 persisted in cattle longer than strain 43894 and correlated with its increased internalization by host cells.

DISCUSSION

The most important finding of this work was that a subpopulation of *E. coli* O157:H7 were internalized by epithelial cells at the bovine RAJ mucosa. This finding supports previous observations that not all *E. coli* O157:H7 are killed by phage lysis or by antimicrobials applied topically or fed. In addition, internalization of *E. coli* O157:H7 by cells at the RAJ mucosa may play a role in bacterial persistence in cattle. The conclusion that some bacteria are internalized and survive in bovine rectal epithelial cells was made both from tissue biopsies from culture positive cattle and from *in vitro* culture of bovine rectal epithelial cells. To accomplish the latter, primary cell culture techniques were adapted to culture epithelial cells from the RAJ mucosa of adult cattle.

The architecture of the bovine RAJ mucosa is comprised of epithelial cells lining crypts and the area is rich in lymphoid follicular tissue (Naylor et al., 2003). The intestinal epithelial cells at this site are rapidly dividing and originate from progenitor cells at the base of the crypt, migrate up and out of the crypt onto the luminal surface of the mucosa where they are shed (Magnuson et al., 2000). Estimates are that the epithelial cells lining the terminal rectum turnover every 4 days as older cells are sloughed and newly dividing cells emerge from the crypts (Falk et al., 1998; Sheng et al., 2006b). Also, the average duration individual animals carry *E. coli* O157:H7 is 1 month (Besser et al., 1997). Cattle carrying *E. coli* O157:H7 are colonized at this tissue site and bacterial cells have been characterized in close association with the mucosa, attached to the surface of the host cells (Naylor et al., 2003). The parameters that trigger clearance or persistence in the carriage of *E. coli* O157:H7 are not fully understood but likely involve a dynamic between bacterial attachment to host cells, bacterial division, host cell division, and host cell turnover. *E. coli* O157:H7 internalization and survival in epithelial cells likely adds to this host:microbe interaction. In this study, the bacteria were present deep in the crypts and were able to survive gentamicin treatment of biopsied tissue that kills all extracellular bacteria. Also, *E. coli* O157:H7 attached to, were internalized by, and survived in cultured cells (PBRE) from this site.

Most pathogenic types of *E. coli* involved in gastrointestinal infections are able to invade cultured epithelial cells (Nataro and Kaper, 1998). Our findings showed that for *E. coli* O157:H7, the efficiency of internalization differed among the strains we tested. The uptake of ATCC 43895 by the PBRE cells was 19.38% of the original inoculum. This level is ~100-fold higher than the other *E. coli* O157:H7 strains tested (**Table 3**) and was at a level similar to previous studies done with a variety of cell lines (Matthews et al., 1997; Dibb-Fuller et al., 2001; Uhlich et al., 2002, 2009; Abu-Ali et al., 2010). Also, ATCC 43895 was more proficient at forming biofilm compared to the other

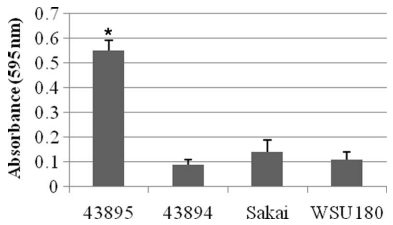

FIGURE 7 | *Escherichia coli* O157:H7 biofilm formation in crystal violet assays. The *E. coli* O157:H7 strains indicated were incubated at 37°C in MSM containing 4% glucose for 24 h in 96-well microtiter plate under stationary condition. Biofilm formation was measured by the absorbance of solubilized dye in 95% ethanol at 595 nm. The assays were performed six times for each isolate. All values are means and SE. Significant difference is indicated by the asterisk ($P < 0.05$).

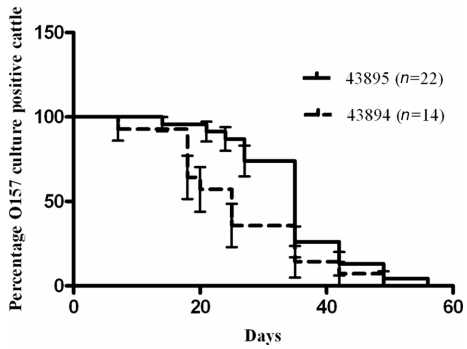

FIGURE 8 | Differential persistence of *E. coli* O157:H7 in cattle. Cattle were challenged with 43895 ($n = 22$) or 43894 ($n = 14$) using a single rectal application of 10^7 CFU of bacteria. Rectal swab samples from each animal was taken twice a week and cultured for *E. coli* O157:H7 until animals cleared the carriage of the bacteria. Percent survival indicated the percent of the cattle that were O157 culture positive. Survival time (duration of carriage) was defined as the number of days animals were cultured positive with *E. coli* O157:H7 and the *P* value was calculated by the Log-rank (Mantel–Cox) test.

strains tested. These two phenotypes of enhanced host cell internalization and biofilm formation may be related and may contribute to the duration bacterial strains are carried by cattle. This idea is supported by the retrospective analysis of cattle challenged with a single rectal application of *E. coli* O157:H7 43895 or 43894 that showed strain 43895 persisted in cattle longer than strain 43894.

Bacterial internalization is associated with the rearrangement of the host cell cytoskeletal structure resulting in endocytosis of the pathogen (Goosney et al., 1999; Donnenberg, 2000). Most invasive enteric bacteria including *Salmonella*, *Shigella*, *Listeria*, and *Yersinia* spp. (Falkow et al., 1992; Finlay and Cossart, 1997; Mengaud et al., 1996) trigger largely microfilament-dependent entry pathways. Invasion of EPEC cells in tissue culture is inhibited by cytochalasin D, colchicines, and by broad specificity Rho GTPase inhibitors (Donnenberg et al., 1990; Ben-Ami et al., 1998). In contrast, *E. coli* O157:H7 internalization was inhibited by cytochalasin D (**Figure 5**) but not by other eukaryotic cell inhibitors. This suggests that *E. coli* O157:H7 uptake by the bovine epithelial cells was through a microfilament-dependent pathway. These data are in agreement with previous studies using immortal cell lines (Matthews et al., 1997) and indicate that EPEC and EHEC exploit distinct host cell processes to stimulate their uptake.

In addition to the actin-dependent host cell microfilament activity required for *E. coli* O157:H7 internalization by epithelial cells, bacterial factors play a role because strain differences in the rate of internalization were found. Tir was not required for efficient uptake of the more efficiently internalized ATCC 43895 strain, but its deletion affected the less efficiently internalized ATCC 43894, although this could be related to the differences in the number of internalized cells or a unique change in the association with the PBRE cells that affected the internalization rate. Close examination of the electron microscopy images of O157-infected PBRE cells showed the interaction between 43895 and PBRE cells was associated with microvilli-like extensions projecting from the host cell membranes and surrounding the bacteria (**Figure 3B**). In addition, Transmission electron microscopy of O157-infected PBRE cells showed bacteria enclosed by membrane-bound vacuoles. This suggests that the localized membrane elongations, which may be induced when the bacteria come into contact with the epithelial cell surface, play a role in the uptake by the PBRE cells. Also, EM images of cross sectioned cells showed >10 intracellular 43895/epithelial cell. This number of intracellular bacteria is comparable with numbers previously reported for other enteric bacterial pathogens such as *Shigella flexneri* (Sansonetti et al., 1986) and *Salmonella typhimurium* (Leung and Finlay, 1991). Invasion of eukaryotic cells is known to be an important survival mechanism of these enteric pathogens (Finlay and Falkow, 1997). However, the internalization of O157 by RAJ epithelial cells is not associated with disease in cattle.

In conclusion, the internalization and survival of a subset of *E. coli* O157:H7 by epithelial cells at the bovine RAJ contributes to the host:microbe interaction. Further study will be required to determine the extent to which internalization contributes to persistence and/or the bovine immune response to this human pathogen and the mechanism of *E. coli* O157:H7 internalization by bovine epithelial cells.

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Proteolytic cleavage of NF-κB p65: a novel mechanism for subversion of innate immune signaling by pathogenic *E. coli*

Stephanie R. Shames[1,2] and B. Brett Finlay[1,2,3]*

[1] *Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada*
[2] *Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada*
[3] *Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada*

**Correspondence: bfinlay@interchange.ubc.ca*

Enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) are attaching and effacing (A/E) bacterial pathogens that cause severe diarrheal disease. An integral mechanism in the virulence strategy of A/E pathogens is the ability to subvert innate immune signaling by down-regulation of interleukin (IL)-8, a pro-inflammatory chemokine that functions to recruit neutrophils to sites of infection (Vallance and Finlay, 2000). Expression of IL-8 is stimulated by several pro-inflammatory stimuli, which interact with cellular receptors and initiate signaling cascades terminating in the expression of the IL-8 gene (Hoffmann et al., 2002). Necessary for IL-8 gene expression is a nuclear factor-κB (NF-κB) dimer containing p65 (Rel-A). In resting cells, NF-κB is held inactive in the cytosol by an inhibitor of κB (IκB); however, NF-κB-activating stimuli facilitate phosphorylation of IκB by an IκB kinase (IKK) and subsequent degradation of IκB (Hoffmann et al., 2002). Liberated NF-κB dimers translocate to the nucleus where they interact with κB-containing enhancers and facilitate gene expression (Hoffmann et al., 2002).

Enteropathogenic *Escherichia coli* and EHEC rely on a type-III secretion system (T3SS) to dampen inflammatory signaling (Vallance and Finlay, 2000). Recently, several studies have demonstrated a role for the non-LEE encoded (Nle) effectors NleB, NleC, NleE, NleH1, and NleH2 in dampening NF-κB-mediated gene expression (Gao et al., 2009; Nadler et al., 2010; Newton et al., 2010; Royan et al., 2010; Yen et al., 2010; Baruch et al., 2011). NleB and NleE stabilize IκBα and thus limit p65 translocation to the nucleus, a mechanism which is shared by the T3SS effector OspZ from *Shigella flexneri* (Nadler et al., 2010; Newton et al., 2010). NleH1 and NleH2 both dampen NF-κB-mediated gene expression, but only NleH1 was shown to mediate this by decreasing nuclear abundance of ribosomal protein S3 (Gao et al., 2009; Royan et al., 2010). Four independent studies have recently shown that NleC is a metalloprotease that enzymatically degrades p65 (Yen et al., 2010; Baruch et al., 2011; Muehlen et al., 2011; Pearson et al., 2011). All four studies demonstrate that an *nleC* null (Δ*nleC*) strain of EPEC could not fully suppress NF-κB activity or IL-8 secretion to levels of wild-type (WT) EPEC. Ectopic expression of NleC could also suppress NF-κB activity and IL-8 secretion (Yen et al., 2010; Baruch et al., 2011; Muehlen et al., 2011; Pearson et al., 2011). All four studies identified a metalloprotease domain within NleC as essential for its function within host cells.

NleC is a zinc-dependent protease with a canonical HEXXH motif (Yen et al., 2010; Baruch et al., 2011; Muehlen et al., 2011). Although NleC is the first type-III secreted Zn-metalloprotease to be identified, other virulence factors have also been shown to degrade p65 to dampen the inflammatory response in the host. *Chlamydia trachomatis* and *C. pneumoniae* both utilize a T3SS to secrete chlamydial protease-like activity factor (CPAF) from chlamydial inclusions into the host cell cytosol (Christian et al., 2010). CPAF functions to degrade p65 and can dampen pro-inflammatory signaling in *Chlamydia* infected cells (Christian et al., 2010). Since *Chlamydia* species are obligate intracellular pathogens, it is not surprising that they encode virulence factors to dampen immune signaling. CPAF does not exclusively target p65 suggesting that NleC may also have other targets in the host cell.

The *nleC* gene is highly conserved between EPEC, EHEC, and *C. rodentium*, a natural A/E pathogen of mice, suggesting that it has a similar function in all three pathogens. The ability of NleC to dampen the release of IL-8 in culture would suggest that a *C. rodentium* Δ*nleC* strain would cause increased inflammation and colitis in murine hosts. This is an attractive theory; however, NleC has little impact on *C. rodentium* colonization and colon weight *in vivo* (Kelly et al., 2006). Use of a *C. rodentium nleC*/*nleE* double knockout would likely provide additional insight into the impact of NF-κB inhibition *in vivo*. NleC is the first p65-targeting metalloprotease to be identified in the effector arsenal of an extracellular pathogen. Recent insights into the function of NleC may aid in elucidating functions for other effector proteins in other pathogens. Understanding the role of effector proteins will further our understanding of their role in virulence and the pathogenic strategy of disease-causing bacteria.

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The evolution of virulence in non-O157 Shiga toxin-producing *Escherichia coli*

Brian K. Coombes[1]*, Matthew W. Gilmour[2,3] and Chelsey D. Goodman[2]

[1] *Department of Biochemistry and Biomedical Sciences, The Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada*
[2] *National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada*
[3] *Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada*

Edited by:
Elizabeth L Hartland, The University of Melbourne, Australia

Reviewed by:
Jorge Giron, University of Florida, USA
Scott Beatson, The University of Queensland, Australia

****Correspondence:***
Brian K. Coombes, Department of Biochemistry and Biomedical Sciences, McMaster University, HSC-4H17, Hamilton, ON, Canada L8N 3Z5.
e-mail: coombes@mcmaster.ca

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic foodborne and waterborne pathogens that are a serious public health concern because they cause outbreaks and the potentially fatal hemolytic uremic syndrome (HUS). The most common STEC serotype associated with human disease is O157:H7, but there is a growing recognition of over 100 non-O157 serotypes that also may result in human illness. Some of these non-O157 STEC strains cause outbreaks and severe disease such as HUS and hemorrhagic colitis, whereas others are associated with only mild diarrhea or with no human disease at all. The relative scarceness of whole genome sequence data for non-O157 STEC has limited the scientific discovery into the genetic basis of these differences in virulence. Uncovering the scope of genetic diversity and phylogeny of the non-O157 STEC through targeting sequencing of clinically relevant isolates will offer new biological insight to the pathogenic behavior of these emerging pathogens. These approaches would also enable molecular risk assessment strategies to rapidly identify and respond to emerging non-O157 STEC that pose a serious public health risk to humans.

Keywords: non-O157 STEC, pathogenesis, genetics, parallel evolution, genome sequencing

NOT ALL STEC ARE CREATED EQUAL

The first outbreak of Shiga toxin-producing *Escherichia coli* (STEC) was recorded in the United States in 1982 (CDC, 1982) and was caused by a clone of serotype O157:H7. A retrospective survey of older culture collections from before the 1980s indicated that this pathogen was a relative newcomer on the scene with a rare prevalence (Griffin and Tauxe, 1991). Additional outbreaks of O157:H7 STEC occurred successively in the UK, continental Europe, Africa, and New Zealand over the next decade and outbreaks associated with contaminated meat, produce and water became more commonplace. In short order, O157:H7 STEC became well-known for its ability to cause outbreaks and hemolytic uremic syndrome (HUS), a serious infection complication involving acute renal failure, hemolytic anemic, and thrombocytopenia. With the awareness of the clinical significance of O157:H7 STEC came an appreciation that non-O157 STEC serotypes were variably associated with human infections as well (Johnson et al., 2006; Bettelheim, 2007). About 150 non-O157 serotypes have now been attributed to sporadic and epidemic human infections (Gould et al., 2009), presenting with a wildly variable spectrum of disease ranging from watery or bloody diarrhea, to HUS and even death. By definition, all of these STEC contain at least one Shiga toxin gene (*stx*), encoding the requisite molecule giving rise to HUS complications following infection (Karmali et al., 1983). However, while some non-O157 STEC produce disease symptoms indistinguishable from O157:H7 infections it is clear that not all non-O157 STEC have the capacity to cause HUS, outbreaks, or even any human infections at all. This presents a significant dilemma for practitioners of clinical medicine and public health to identify high-risk STEC at the interfaces between humans, animals, food products, and the environment. The differences in host genetics notwithstanding, it is fair to say at this point that the genetic basis underlying the varied clinical spectrum of disease in non-O157 STEC infections remains unsolved.

The ability to identify the non-O157 STEC that pose a risk to the human population using a molecular genetic approach would be a boon to clinical medicine where the diagnosis of non-O157 is challenging at best, or worst, not attempted at all (Bettelheim, 2007). It would also represent a significant public health achievement. In fact, almost 15 years ago the World Health Organization called the rapid identification of virulent non-O157 STEC a public health priority (WHO, 1998), yet few initiatives have occurred since the call to advance this agenda.

MOBILE ELEMENTS ARE A SOURCE OF GENETIC NOVELTY

Virulence in bacterial pathogens can be attained by acquisition of mobile genetic elements such as prophages, transposons, plasmids, and genomic islands (Lawrence, 2005). One class of genomic island, called pathogenicity islands (PAI), contains virulence genes that are involved in host colonization and disease (Dobrindt et al., 2004). PAIs constitute a flexible gene pool contributing to evolution and virulence potential and can be used as a genetic signature of new and emerging pathogens. In STEC for example, the locus of enterocyte effacement (LEE) is a chromosomal PAI encoding a type III secretion system (T3SS) necessary for the attaching and effacing lesion that is characteristic of disease associated with this organism. Notably, the T3SS is a key genetic determinant of both colonization and persistence in animal reservoirs and for virulence in humans following zoonotic transmission

(Dziva et al., 2004; Coombes, 2009). Given that ruminant animals are a natural host for STEC, such data provides persuasive arguments that the origins of STEC type III secretion might have little to do with virulence, but rather its use by modern day pathogens as a virulence factor is a convenient upshot of its selection in a different ecological setting. Levin (1996) described this evolutionary trajectory as "coincidental evolution," where the factors involved in virulence evolved for a purpose other than virulence *per se*. Genetic screens indicate that the substrates of the T3SS, called effectors, contribute to persistence in cattle of both O157:H7 and non-O157 STEC (Van Diemen et al., 2005), and the individual contribution of some of these effector molecules in colonization, transmission and virulence has been quantified (Wickham et al., 2007; Croxen and Finlay, 2010). Such data highlight a clinically relevant link between colonization in animals and human virulence that is influenced by the repertoire of type III secreted substrates in the associated strain. This information has important implications for public health because the persistence of type III secretion-competent strains in animal populations would contemporaneously increase the opportunities for zoonotic, foodborne, and environmental transmission of the most virulent strains to humans (Coombes, 2009).

Genome sequences of O157:H7 STEC have expanded the number of mobile genetic elements well beyond the prototypical LEE PAI (Perna et al., 2001). These include many of the O-islands (OI) that were originally defined as lineage specific segments present in O157:H7 *E. coli* but absent from non-pathogenic *E. coli* K12. Many of these have origins in bacteriophages that when integrated as prophages into the bacterial chromosome, endowed that STEC with new genetic information that could eventually be used to modulate colonization and infection biology in humans. The individual gene content of some of these O-islands has been linked to the epidemic potential and disease severity associated with non-O157 STEC (Coombes et al., 2008). The precise contribution of all mobile genetic elements to STEC virulence and fitness, especially non-O157 STEC, is unknown in most cases and a comprehensive investigation of virulence determinants has not been undertaken for the non-O157 STEC.

PUBLIC HEALTH PATHOGENOMICS AND THE CONCEPT OF SEROPATHOTYPE

Recognizing that the virulence potential of non-O157 STEC was likely due to heritable genetic traits, Karmali et al. (2003) established a seropathotype classification framework that considers serotype association with prior human epidemics, HUS, and diarrhea. For example, seropathotype (SPT)-A comprises O157:H7 and O157:NM which are the most common causes of outbreaks and HUS in most countries; SPT-B strains are also associated with outbreaks and HUS, but less frequently than SPT-A; SPT-C strains are associated with sporadic HUS but not epidemics; SPT-D strains are associated with diarrhea but not with outbreaks or HUS, and SPT-E comprises multiple STEC serotypes that have never been associated with human disease. Although seropathotype memberships are based on real world clinical associations (or absence thereof) with disease, the real genetic basis behind the distribution of serotypes into a seropathotype category is not known. It can be reasonably assumed to be due to the acquisition of virulence genes and their continued maintenance as part of the flexible gene pool in a strain's genome, but the genomic landscape of non-O157 STEC spanning the various SPT classes has not yet been charted to address this hypothesis in a comprehensive manner. Although informative as an *ex post facto* determinant of virulence potential, the mobile nature of virulence genes exposes a limitation of SPT classification as a prognostic indicator of microbial risk. Because many virulence genes are acquired by horizontal gene transfer, it is possible (and perhaps likely) that STEC that have never been associated with human disease (i.e., SPT-E) could acquire mobile genetic elements through parallel evolution that convert them into a more virulent strain capable of human infections. In this context, identifying the genetic complement involved in human infections and understanding the complex biology of this host–pathogen interaction is a major research goal.

UNDERSTANDING NON-O157 STEC VIRULENCE

The notion of parallel evolution is well-known for pathogenic *E. coli* (Donnenberg and Whittam, 2001; Croxen and Finlay, 2010). This idea was put forward over a decade ago (Reid et al., 2000) and revisited recently following the first ever sequencing of three non-O157 STEC associated with human disease, serotypes O26, O111, and O103 (Ogura et al., 2009). These seropathotype B strains are emerging pathogens with clinical importance in many countries as a source of outbreaks and HUS complications following infection (European Commission, 2007). The genome sequences for O26-, O111-, and O103-STEC affirmed that, just as O157:H7 arose multiple times in a series of genetic steps from a pathogenic O55:H7 ancestor (Wick et al., 2005), so too have pathogenic non-O157 STEC arose from the independent acquisition of mobile genetic elements harboring common virulence genes. From what common ancestor, if any, these derive from is unknown. In these SPT-B strains, the T3SS and associated elements appear to have been constructed independently by lambdoid prophages and other integrative elements that others have previously shown to be involved in the stepwise evolution of O157:H7 (Tobe et al., 2006; Ogura et al., 2009). The stepwise acquisition of about 30 known T3SS effectors in STEC, chiefly the horizontally acquired non-LEE-encoded *nle* genes, are particularly interesting when considered in the context of selective forces driving their maintenance in the genome.

It is known that unlinked effectors can work in concert with one another to produce a desired effect on the host cell (Tree et al., 2009; Newton et al., 2010), or have functions that oppose host phenotypes induced by other effectors (Simovitch et al., 2010). A delivery order for effectors has also been suggested, with the translocated intimin receptor, Tir, sitting atop a delivery hierarchy needed for efficient secretion of later type III cargos (Thomas et al., 2007). Also implicit is that the positive selection of newly landed effector genes would take place in a genetic background already competent for the T3SS, since bacteria would presumably find little use for secreted cargo without the secretion machinery needed to deliver it. It is currently unclear whether there is an acquisition hierarchy for the available effector repertoire, or whether T3SS effector genes can be maintained for some time under neutral selection in the genome of strains lacking effector partners or the core T3SS itself. What dictates the "order beneath chaos" (Lawrence and Hendrickson,

2005) of STEC genome evolution? How might evolutionary intermediates be selective, and in what environmental or animal niche does this selection take place? To get a handle on these questions, more sequence information is needed. Sequence information for additional non-O157 STEC that span the clinical divide from the worst offenders (SPT-B) to the seemingly benign (SPT-E) will be the first step in uncovering the provenance of pathogenic behavior in this emerging group of bacteria.

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A fresh look at the type III secretion system: two-step model of effector translocation in pathogenic bacteria

Ana Victoria C. Pilar and Brian K. Coombes*

Department of Biochemistry and Biomedical Sciences, Michael G. De Groote Institute for Infectious Disease Research, McMaster University, Hamilton, ON, Canada
**Correspondence: coombes@mcmaster.ca*

A commentary on

Translocation of surface-localized effectors in type III secretion
by Akopyan, K., Edgren, T., Wang-Edgren, H., Rosqvist, R., Fahlgren, A., Wolf-Watz, H., and Fallman, M. (2011). Proc. Natl. Acad. Sci. U.S.A. 108, 1639–1644.

Acquisition of genetic elements such as virulence plasmids or pathogenicity islands (PI) by horizontal gene transfer can endow pathogenic bacteria with an arsenal of virulence factors that promote bacterial survival and replication within their hosts. Despite the differences in the host organisms and pathology caused by important pathogenic bacteria such as *Escherichia coli*, *Yersinia*, *Salmonella*, and *Shigella*, a common virulence mechanism exists in the form of a needle-like structure that translocates bacterial proteins into host cells to hijack the host machinery and modulate the host immune response (Ghosh, 2004).

Enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) belong to a family of attaching and effacing (A/E) pathogens responsible for diarrheal diseases in humans and animals. The diseases are characterized by the effacement of the intestinal microvilli, bacterial colonization, and attachment on pedestals induced by localized actin polymerization upon contact with enterocytes and disruption of tight junctions (Dean and Kenny, 2009; Croxen and Finlay, 2010). A type III secretion system (T3SS) encoded by the locus of enterocyte effacement (LEE) secretes proteins called effectors to form A/E lesions in the host and subvert various host processes such as disruption of the host cytoskeletal network and modulation of the host innate immune signaling (Sharma et al., 2006; Ruchaud-Sparagano et al., 2007; Khan et al., 2008). *Yersinia* employs the plasmid-encoded Ysc-Yop T3SS to deliver effectors called Yops (*Yersinia* outer proteins) to the host cytosol to paralyze phagocytes and block bacterial uptake (Cornelis et al., 1989; Rosqvist et al., 1990). This tactic of evading the host immune response ensures an environment conducive for the lifestyle of *Yersinia*. In contrast, *Salmonella* possesses two PI encoding distinct T3SS called SPI-1 and SPI-2. During invasion, the SPI-1 secretion apparatus deploys effectors to the host cell milieu to promote phagocytosis (Galán, 1999) while the SPI-2 T3SS activity creates a niche for replication and survival of *Salmonella* within target cells (Cirillo et al., 1998). The components of the T3SS are generally conserved among Gram-negative bacteria and even heterologous effectors can be secreted in another host bacteria such as the case of the *Yersinia* effector YopE expressed in *Salmonella enterica* serovar Typhimurium (Rosqvist et al., 1995).

The *Yersinia* injectisome consists of a membrane-spanning basal body and a hollow conduit of YscF polymers through which effectors transit for secretion (Hoiczyk and Blobel, 2001). Dedicated T3SS chaperones bind to their cognate effectors and keep them in a locally unfolded, secretion-competent state (Ghosh, 2004). The chaperone-effector interaction is thought to provide specificity for effector docking on the secretion apparatus. The N-terminal domain or the 5′ end of most secreted effectors contains the signal sequence for secretion, translocation, and chaperone binding (Sory et al., 1995; Miao and Miller, 2000). However, there is no clear consensus sequence for the signals due to the degeneracy of the sequences at the amino acid or RNA level (Ghosh, 2004). How these signals are recognized by the secretion apparatus is not well understood but differential signal recognition by the chaperones or translocon components is thought to be the basis for the hierarchical secretion of effectors (Lara-Tejero et al., 2011; Osborne and Coombes, 2011). A defined order of secretion of effectors ensures that effector functions are activated in a spatial and temporal manner. A recent study revealed a cytoplasmic complex made up of SpaO/OrgA/OrgB that functions as a platform for sorting chaperone-effector pairs prior to secretion (Lara-Tejero et al., 2011). Differential binding of the specific chaperones to the complex leads to the sequential loading of substrates. The translocators YopB and YopD are needed to complete the translocation of effectors across the host cell membrane and deletion of these translocators results in the extracellular secretion but not translocation of effectors into the host cytosol (Håkansson et al., 1996; Neyt and Cornelis, 1999). In *Yersinia*, the first ~15 amino acids at the N-terminus is sufficient for secretion but not for translocation of the effectors leading to the conclusion that the presence of YopB/YopD and a distinct translocation signal are required for proper effector translocation (Sory et al., 1995). In the absence of translocators, secretion of effectors can be induced by growing bacteria in media that mimic environmental cues for T3SS activation such as low calcium, phosphate or magnesium (Michiels et al., 1990; Yu et al., 2010). Hence, although secretion and translocation are both necessary for infection, these events have different regulatory and structural requirements.

YopB/D, as well as the related translocators in *E. coli* (EspB/D), *Shigella* (IpaB/C/D), and *Salmonella* (SipB/C/D), contain hydrophobic domains and are proposed to form a pore by inserting into the host cell membrane (Ghosh, 2004). However, direct evidence for effectors being transported through this pore is lacking. In the one-step microinjection model, the effectors are injected directly by the T3SS into the host cytosol. However, one issue with this model lies in the structural and functional relationship of the translocators and the injectisome. It still remains to be elucidated whether the injectisome itself actually pierces the host cell membrane or the translocators act as the terminal connection of the injectisome to the target cells by creating a membrane pore (Hoiczyk and Blobel, 2001; Cornelis, 2002).

A recent paper by Akopyan et al. (2011) aimed to elucidate the translocation mechanism of the T3SS. They demonstrated that *Y. pseudotuberculosis* effectors localized to the bacterial surface are translocated by the T3SS. They first observed that the effectors YopE and YopH and the translocator YopD were evenly distributed on the bacterial surface prior to host cell contact (Schesser et al., 1996; Akopyan et al., 2011). Interestingly, previous studies also found secreted Ipa effectors on the surface of *Shigella* before injection into the host cell (Watarai et al., 1995). The significance of these findings is unclear but it was proposed that the extracellular effectors serve a protective role for the pathogen against the onslaught of the host immune attack (Schesser et al., 1996). Mutational analysis of the N-terminal region of YopE showed that distinct domains are needed for secretion, surface localization, and translocation across the host cell membrane. YopE disrupts the host cytoskeleton to prevent phagocytosis and having a distinct pool of surface-localized YopE could provide an early protection for the bacterium before host cell contact (Schesser et al., 1996).

Akopyan et al. (2011) further investigated the role of surface-localized effectors by demonstrating that bacteria coated with recombinant purified YopH induced YopH-mediated inhibition of early calcium response in neutrophils during infection. These results were dependent on a functional T3SS and the translocators YopB and YopD. To prove that the response was due to the translocation of the surface-localized effector, they coated bacteria with a recombinant YopH-Bla fusion protein and showed β lactamase activity in the host cytosol after infection. Truncations at the N-terminal region of YopH-Bla showed that distinct domains are required for effector secretion and translocation. Coating of bacteria with $YopH_{1–17}$-Bla did not result in the translocation of the mutant effector, substantiating previous studies that showed expression of the first 17 amino acids of YopH is sufficient for T3SS-mediated secretion of the effector but not translocation into the host cytosol (Sory et al., 1995; Akopyan et al., 2011). On the other hand, a distinct signal sequence found between amino acids 18 to 49 was required for effector translocation, as recombinant $YopH_{18–49}$-Bla or $YopH_{18–99}$-Bla used to coat bacteria were exported into the host cell. However, these YopH mutants were not secreted when expressed in bacteria and in the absence of surface localization, strongly suggesting that effector secretion, and translocation are two separate events (Boyd et al., 2000).

Based on the findings of this study, the authors proposed a two-step model in which formation of an effector-translocator complex represents the intermediate step between effector secretion by the injectisome and translocation through the pore formed by YopB and YopD. In this model, the N-terminal translocation domain is critical for the formation of this intermediate and could explain why separate N-terminal secretion and translocation domains are needed for efficient delivery of T3SS substrates. However, direct evidence of the intermediate is still lacking and whether the binding of the effector to YopB/D occurs through the translocation domain has yet to be determined. It is hypothesized that YopB/YopD block the membrane pore to prevent host membrane damage after infection (Viboud and Bliska, 2001). It would be interesting to study whether the formation of the effector-translocator intermediate induces conformational changes in YopB/D to enable opening of the pore.

Akopyan et al. (2011) proposed that pre-formed translocation intermediates could be found on the bacterial surface or bacteria-host membrane interface prior to translocation. How does surface-localization relate to the observed hierarchical secretion of effectors? It is possible that some of the surface-localized effectors compete with the pool of T3SS substrates originating directly from the bacterial cytosol for binding with the translocators. The rest of the surface-localized effectors could function in protecting the pathogen. This dual role for T3SS effectors, as well as the presence of an available pool of extra- and intracellular T3SS substrates, might be beneficial for the pathogen as this primes the bacteria during its transit from the extracellular environment to target tissues. Alternatively, the secretion hierarchy could be maintained by secreting early-acting effectors that are then localized to the bacterial surface in preparation for host cell contact. In *Salmonella*, it has been shown that expression of SPI-2 genes occurs before invasion of intestinal epithelium (Brown et al., 2005). Whether effectors from both T3SS are localized to the surface of the bacteria before invasion is not known. The possibility for surface-localized effectors in *Salmonella* was supported by experiments showing *S.* Typhimurium coated with recombinant YopH also translocated the effector into host cells in a SPI-1 T3SS-specific manner (Akopyan et al., 2011). Thus, the two-step model challenges our view of the translocation mechanism of the T3SS and provides a fresh look at the events occurring at the pathogen-host cell interface. Future work on the identification and translocation of surface-localized effectors in *E. coli, Salmonella,* and other pathogenic Gram-negative bacteria will provide needed insight into the two-step translocation mechanism of T3SS. It will particularly interesting to see whether this mechanism of effector translocation also occurs in alternative host settings, particularly the host-pathogen interactions leading to commensalism of EHEC and EPEC in animals.

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Surface structures involved in plant stomata and leaf colonization by Shiga-toxigenic *Escherichia coli* O157:H7

Zeus Saldaña[1], Ethel Sánchez[2], Juan Xicohtencatl-Cortes[3], Jose Luis Puente[4] and Jorge A. Girón[1]*

[1] *Department of Molecular Genetics and Microbiology, Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA*
[2] *Centro de Investigación en Estructuras Microscópicas, Universidad de Costa Rica, San José, Costa Rica*
[3] *Laboratorio de Bacteriología Intestinal, Hospital Infantil de México Federico Gómez, México D.F., México*
[4] *Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México*

Edited by:
Adel M. Talaat, University of Wisconsin Madison, USA

Reviewed by:
Michael Konkel, Washington State University, USA
Jay C. D. Hinton, Trinity College Dublin, Ireland

****Correspondence:***
Jorge A. Girón, Department of Molecular Genetics and Microbiology, Emerging Pathogens Institute, University of Florida, 2055 Mowry Road, PO Box 100009, Gainesville, FL, 32610, USA.
e-mail: jagiron@ufl.edu

Shiga-toxigenic *Escherichia coli* (STEC) O157:H7 uses a myriad of surface adhesive appendages including pili, flagella, and the type 3 secretion system (T3SS) to adhere to and inflict damage to the human gut mucosa. Consumption of contaminated ground beef, milk, juices, water, or leafy greens has been associated with outbreaks of diarrheal disease in humans due to STEC. The aim of this study was to investigate which of the known STEC O157:H7 adherence factors mediate colonization of baby spinach leaves and where the bacteria reside within tainted leaves. We found that STEC O157:H7 colonizes baby spinach leaves through the coordinated production of curli, the *E. coli* common pilus, hemorrhagic coli type 4 pilus, flagella, and T3SS. Electron microscopy analysis of tainted leaves revealed STEC bacteria in the internal cavity of the stomata, in intercellular spaces, and within vascular tissue (xylem and phloem), where the bacteria were protected from the bactericidal effect of gentamicin, sodium hypochlorite or ozonated water treatments. We confirmed that the T3S *escN* mutant showed a reduced number of bacteria within the stomata suggesting that T3S is required for the successful colonization of leaves. In agreement, non-pathogenic *E. coli* K-12 strain DH5α transformed with a plasmid carrying the locus of enterocyte effacement (LEE) pathogenicity island, harboring the T3SS and effector genes, internalized into stomata more efficiently than without the LEE. This study highlights a role for pili, flagella, and T3SS in the interaction of STEC with spinach leaves. Colonization of plant stomata and internal tissues may constitute a strategy by which STEC survives in a nutrient-rich microenvironment protected from external foes and may be a potential source for human infection.

Keywords: plant colonization, stomata, STEC, O157:H7, pathogenesis, spinach, T3SS, pili

INTRODUCTION

Human infections due to Shiga-toxigenic *Escherichia coli* (STEC) O157:H7 strains range from mild diarrhea to bloody diarrhea or hemorrhagic colitis, which can develop into the potentially fatal hemolytic uremic syndrome or HUS (Karmali et al., 1983; Riley et al., 1983). STEC is a significant dilemma in the realm of food-borne disease, exemplified in recent episodes of diarrheal disease in various regions of the world associated with consumption of contaminated food, water, milk, fruit juices, and leafy green vegetables such as alfalfa, sprouts, arugula, spinach, and lettuce (Michino et al., 1999; Sivapalasingam et al., 2004; Wendel et al., 2009). The precise mechanisms by which STEC is introduced into agricultural products are not fully understood. The bacteria live as commensal organisms in farm and several wild animals and may represent a major source of contamination of water and agricultural produce for human consumption (Solomon et al., 2002; Mootian et al., 2009). For example, recent outbreaks of diarrheal illness associated to spinach consumption were linked to contamination with feces from STEC-harboring wild pigs (CDC, 2006; Jay et al., 2007). Insects *(e.g., filth flies)* have been implicated as transmission vectors in plant infection by STEC (Talley et al., 2009).

The phyllosphere supplies an important bio-niche for a significant number and wide range of commensal bacterial species as well as for plant and human pathogens (Lindow and Brandl, 2003). Pathogen-associated molecular patterns (PAMPs) such as flagella and lipopolysaccharide (LPS) from Gram-negative bacteria interact with specific target receptors on guard cells to elicit an innate response resulting in the closure of stomata on the *Arabidopsis* leaf surface (Melotto et al., 2006). Illness-producing bacteria have evolved strategies to overcome plant defenses and to colonize the surface of leaves and deeper tissues of the phyllosphere. They use wounds and natural openings like stomata, hydathodes, and lenticels as natural gateways for passage into internal plant tissues where they grow and begin to cause disease (Hirano and Upper, 2000; Melotto et al., 2006). *Pseudomonas syringae*, a pathogen of hundreds of plant species utilizes its secretion (type 3 secretion system, T3SS) and an array of effector molecules to successfully suppress plant innate mechanisms of defense and kill its host (Chang et al., 2005; Chisholm et al., 2006). *Listeria monocytogenes* uses flagella for attachment to radish tissue and *Klebsiella, Enterobacter, and Pseudomonas* produce fimbriae that mediate attachment to plant surfaces (Korhonen et al., 1986; Vesper, 1987; Gorski et al., 2003). It was suggested that *E. coli* O157:H7 is able to exist as a component

of the phyllosphere microbial community both as epiphytes on leaf surfaces and endophytes within leaf tissues. While some authors suggest that *E. coli* O157:H7 could be absorbed from soil to internal plant tissues, others suggest entry through plant roots (Wachtel et al., 2002; Islam et al., 2004; Jablasone et al., 2005; Sharma et al., 2009). *E. coli* O157:H7 can be located and is able to survive in the substomatal cavity and the intercellular spaces of the spongy of leafy greens (Itoh et al., 1998; Solomon et al., 2002; Warriner et al., 2003; Franz et al., 2007). The precise mechanisms of adherence of STEC to plant surfaces, the receptors recognized, and the types of physical interactions participating in this interplay are largely unknown.

Much is known about the mechanisms of interaction of STEC with human epithelial cells. STEC uses its T3SS to inject many effector proteins into intestinal cells that lead to remodeling of the cytoskeleton and consequently to the formation of intestinal attaching and effacing (AE) histopathological lesions. Most of the components required for AE are encoded in the genetic locus of enterocyte effacement (McDaniel and Kaper, 1997). It is well established that the interaction of STEC with human epithelial cells is multi-factorial as it involves the participation of the intimin–Tir complex, secreted proteins, and flagella. Several pili types including, but not limited to, the long polar fimbriae, F9, hemorrhagic coli pilus (HCP), curli, the *E.* coli laminin-binding fimbriae (ELF), and the *E.* coli common pilus (ECP), were associated with adherence to human and animal epithelial cells *in vitro* (Torres et al., 2002; Kaper et al., 2004; Low et al., 2006; Erdem et al., 2007; Rendon et al., 2007; Xicohtencatl-Cortes et al., 2007; Saldana et al., 2009; Samadder et al., 2009). It was reported that the EspA fiber, but not intimin, flagella or pili, was required for attachment of STEC to arugula leaves (Shaw et al., 2008). Recently, we suggested that the T3SS of STEC O157:H7 aids in the colonization of lettuce and spinach (Xicohtencatl-Cortes et al., 2009).

The aim of this study was focused on expanding our knowledge on the interaction of STEC with baby spinach leaves and the role of newly described surface adherence factors in colonization. This effort revealed that STEC uses several surface structures to colonize the leaf surface and gain access to internal tissues and in this way, establish a niche in the plant where bacteria are protected from environmental foes and industrial decontamination. This strategy would ensure survival in the environment and allow transmission to animal reservoirs and to the human host.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE CONDITIONS

Strains used here are described in **Table 1**. Bacteria were routinely grown overnight at 37°C with aeration in Luria–Bertani (LB) broth or for induction of virulence factors in Dulbecco's modified Eagle's medium (DMEM) with 0.5% D-mannose (Invitrogen). Except for the intimin (*eae*), quorum sensing (*qse*B), and Tir (*tir*) mutants constructed here, all of the remaining mutants and complemented strains employed have been reported previously by our laboratories or other researchers.

CONSTRUCTION OF BACTERIAL MUTANTS

The *tir, eae,* and *qseB* genes in STEC O157:H7 strain EDL933 were deleted by the λ Red recombinase mutagenesis approach as described earlier (Datsenko and Wanner, 2000). The primers employed for mutagenesis are described in **Table 2**. The isogenic mutants were confirmed for the lost genotype by PCR. All mutant derivatives grew at the same rate as the wild-type strain under the conditions tested in this work.

Table 1 | Bacterial strains and plasmids used in this study.

Strains source	Notes	References or source
EDL933	Wild type O157:H7	Riley et al. (1983)
EDL933Δ*escN*	T3SS-ATPase *escN*::km	Jarvis and Kaper (1996)
EDL933Δ*tir*	Tir mutant *tir*::km	This study
EDL933Δ*eae*	Intimin mutant *eae*::km	This study
EDL933Δ*espFu*	*espFu*::cm	Campellone et al. (2004)
EDL933Δ*espP*	*espP*::km	Xicohtencatl-Cortes et al. (2010)
EDL933Δ*fliC*	*fliC*::km	Erdem et al. (2007)
EDL933Δ*qseB*	*qseB*::cm	This study
EDL933Δ*hcpA*	*hcpA*::km	Xicohtencatl-Cortes et al. (2007)
EDL933Δ*ecpA*	*ecpA*::km	Rendon et al. (2007)
EDL933Δ*elfA*	*elfA*::km	Samadder et al. (2009)
EDL933Δ*csgA*	*csgA*::km	Saldana et al. (2009)
EDL933Δ*csgA* (pCsgA)	pCsgA = *csgBA* in pBAD-Topo	Saldana et al. (2009)
EDL933Δ*csgD*	*csgD*::km	Saldana et al. (2009)
EDL933Δ*csgD* (pCP994)	pCP994 = *csgD* in pKK233-2	Saldana et al. (2009)
EDL933Δ*bcsA*	*bcsA*::cm	Saldana et al. (2009)
DH5α	K-12 strain	Our collection
DH5α (pLEE)	LEE plasmid	This study

SPINACH COLONIZATION ASSAYS

Fresh processed baby spinach leaves (cut into 1 cm-diameter pieces) were washed with sterile PBS and placed into 24-well plates containing DMEM with 0.5% D-mannose. The leaves were then infected with approximately 10^7 bacteria from an overnight culture grown in LB broth and incubated at 26°C for specific time periods (0–24 h) or as indicated in the text. The supernatant was then removed and the leaves were washed three times with sterile PBS to remove unbound bacteria. For quantitative assessment of bacterial adherence, spinach leaves infected with wild type or isogenic mutants were placed in 1.5 ml Eppendorf tubes containing glass beads in 1 ml of sterile PBS with 0.3% Triton X-100 and vortexed for 5 min. The supernatant was collected and 10-fold serial dilutions were plated out onto MacConkey-Sorbitol agar plates for determination of colony-forming units (CFUs) after overnight incubation (Xicohtencatl-Cortes et al., 2009). When required, the infected leaves where incubated for 2 h with gentamicin (200 μg/ml) to kill surface-exposed bacteria, and the bacteria inside the leaves tissues surviving this treatment, were counted as described above. All experiments were repeated at least three times in triplicate on different days and the results were expressed as percentage of adherence relative to that of the wild-type strain or as CFUs.

ELECTRON MICROSCOPY STUDIES

For transmission electron microscopy (TEM) infected and mock-infected leaves were washed as described above and fixed with fixative Trumps (Electron Microscopy Sciences). Fixed tissues were

Table 2 | List of primers used in this study.

Primer name	Gene	Function	Primer Sequence 5′ → 3′
K1 f	km	Confirm mutagenesis	GTGTATTGACGGCGTTTATAACTGTGGTATGTGCA
			ACGTCCAGCGTTATGGTGTAGGCTGGAGCTGCTTC
K2 r	km	Confirm mutagenesis	TGTTACCGCATTCACATTACCAGAAGTCGCTTTCC
			CCGTCATATGAATATCCTCCTTAG
H*tir*-H1P1 f	*tir*	Mutagenesis	AATAAAAAGGAGATATTTATGCCTATTGGTAATCTTGGTCATTGTAGGCTGGAGCTGCTTCG
H*tir*-H2P2 r	*tir*	Mutagenesis	AAATGATTATGGATATATTTAGACGAAACGATGGGATCCCGGCATATGAATATCCTCCTTAG
H*eae*-H1P1f	*eae*	Mutagenesis	TTGTGGTGGAGCCATAACATGATTACTCATGGTTGTTATACCTGTAGGCTGGAGCTGCTTCG
H*eae*-H2P2 r	*eae*	Mutagenesis	GCCGGGGTGGTTATGGAATTATTCTACACAAACCGCATAGACCATATGAATATCCTCCTTAG
G250 f	*qseB*	Mutagenesis	TATCGCAGGGATGAAAAAATGCGAATTTTACTGATAGAAGTGTAGGCTGGAGCTGCTTC
G251r	*qseB*	Mutagenesis	CTCACCTAATGTGTAGCCAATACCATGCTCGGTACGAATCATATGAATATCCTCCTTAG
H*tir* f	*tir*	Confirm mutagenesis	GGTTGCTGCAATTTTATTTAT
H*tir* r	*tir*	Confirm mutagenesis	TGAAATAGATCCAATACCAATT
H*eae* f	*eae*	Confirm mutagenesis	AGAATGAAATAGAAGTCGTTG
H*eae* r	*eae*	Confirm mutagenesis	TTGTGAGCATAGTTGTTGCT
G254 f	*qseB*	Confirm mutagenesis	AGGGCCATTACTGCGATTAC
G255 r	*qseB*	Confirm mutagenesis	GAGCGTACTTAACCGCTTGG

F, forward; r, reverse.

processed with the aid of a PelcoBioWave laboratory microwave (Ted Pella). The samples were washed in 0.1 M sodium cacodylate pH 7.2, post fixed with 2% OsO_4, water, washed, and dehydrated in a graded ethanol series 25, 50, 75, 95, 100% followed by 100% acetone. Dehydrated samples were infiltrated in graded acetone/Spurs epoxy resin (30, 50, 70, 100%), and cured at 60°C for 48 h. Cured resin blocks were trimmed, thin sectioned, and collected on Formvar copper slot grids, post-stained with 2% aq. Uranyl acetate and Reynolds' lead citrate. Sections were examined with a Hitachi H-7000 TEM (Hitachi High Technologies America) and digital images acquired with a Veleta 2k × 2k camera and iTEM software (Olympus Soft-Imaging Solutions Corp.). For scanning electron microscopy (SEM), infected and mock-infected leaves were fixed, dehydrated as mentioned above and critically point dried (Bal-Tec CPD030, Leica Microsystems). Samples were mounted on carbon adhesive tabs on aluminum specimen mount, Au/Pd sputter coated (DeskV, Denton Vacuum), and examined with high-resolution field-emission scanning electron microscope (S-4000, Hitachi High Technologies America).

ANTISERA

Rabbit polyclonal antibodies raised against STEC antigens, H7 flagella, O157 LPS, ECP, and curli, were available from previous studies and their specificity has been previously described (Rendon et al., 2007; Saldana et al., 2009; Xicohtencatl-Cortes et al., 2009).

LIGHT AND IMMUNOFLUORESCENCE MICROSCOPY

Infected leaves were washed three times with sterile PBS to remove unbound bacteria and then fixed with 2% formalin/PBS for 20 min. Peels of the lower surfaces of the leaves (epidermis abaxial) were observed by light microscopy to visualize bacteria associated with or within stomata openings as previously described (Melotto et al., 2006). For immunofluorescence microscopy (IFM), leaves fixed with 2% formalin, were incubated for 1 h with primary rabbit antibodies (listed above) followed by 1-h incubation with secondary anti-rabbit IgG Alexa-conjugated antibodies (Molecular Probes). The specimens were visualized under UV light and phase contrast using an Axio Imager1.0 Zeiss microscope.

RESISTANCE TO OZONATED WATER AND CHLORINATED SOLUTIONS

To determine resistance to chlorine treatment, leaves of approximately the same size were placed in Petri dishes and were infected at 26°C for 9 h with approximately 10^7 bacteria from overnight cultures grown in DMEM. After the desired incubation period, the spinach leaves were subjected to 10 min-washes in 10-fold dilutions (0.1–10%) of sodium hypochlorite (chlorine), or 10–20 min exposure to ozonated water. Subsequent to this treatment, the spinach leaves were washed three times with sterile PBS, cut into 1 cm-diameter pieces, and placed in 1.5 ml Eppendorf tubes containing 1 ml of sterile PBS with 0.3% Triton X-100 and glass beads and processed as described above for bacterial counting (Xicohtencatl-Cortes et al., 2009). Statistical analysis was done using Student's *t*-test. The significance level was 5% in all tests ($^*P < 0.05$). The GraphPad software was used.

RESULTS

ROLE OF FLAGELLA AND T3SS IN LEAF COLONIZATION

We sought to investigate the importance of these surface structures in leaf adherence and colonization. Kinetics studies of spinach infections performed between 0 and 8 h assisted by IFM using specific anti-H7 antibodies revealed the compelling presence of flagella on bacteria adhering to the leaf epidermis. Progressive and increasing amounts of flagella H7 were observed correlating with the increasing number of bacteria adhering with respect to time of incubation (**Figure 1**). SEM analysis of wild type versus a genetically modified flagella (non-motile) mutant supported these observations (**Figure 1G**). Comparative quantitative experiments showed that the flagella (*fliC*) and T3SS (*escN*) mutants adhered significantly less than the parental strain confirming a role of these appendages in spinach colonization (**Figure 2A**). We

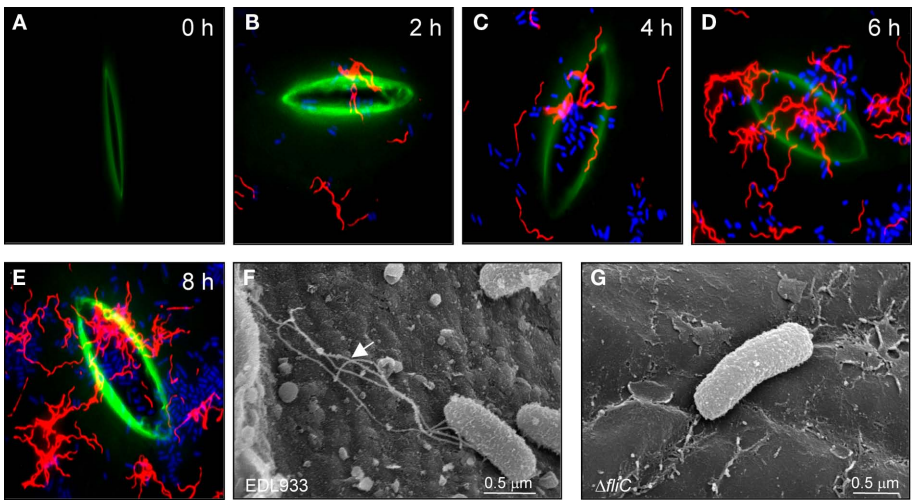

FIGURE 1 | Time-course visualization of flagella on STEC infecting spinach leaves. (A–E), Leaves infected with STEC for 0, 2, 4, 6, and 8 h were processed for IFM using anti-H7 antibodies. Flagella are shown in red, bacteria in blue, and the stomata in green. Images were taken at 60X. **(F)** Scanning electron micrograph showing EDL933 adhering onto the leaf epidermis and displaying flagella (arrow). **(G)** The *fliC* mutant did not show the structures seen in **(F)**.

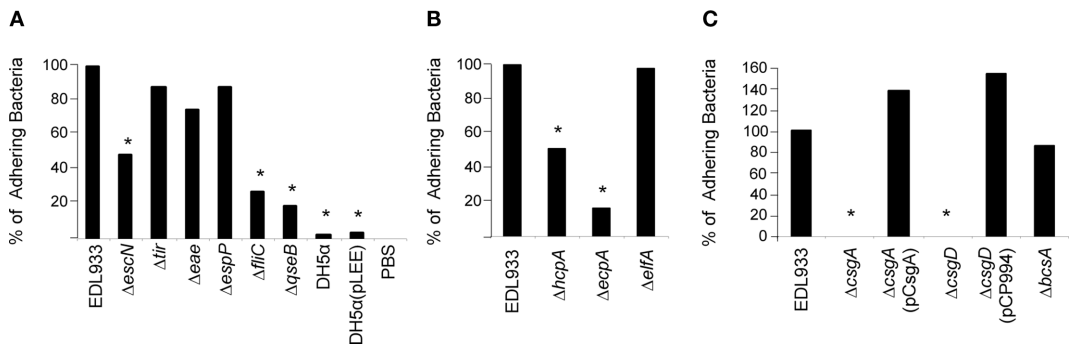

FIGURE 2 | Comparative analysis of spinach colonization by STEC strains after 9 h of infection. (A) EDL933 and isogenic mutants in *escN* (T3SS), *tir* (intimin receptor), *eae* (intimin), *espP* (toxin/adhesin), *fliC* (flagellin), and *qseB* (quorum sensing gene). **(B,C)** EDL933 and isogenic mutants in pilin genes: *hcpA, ecpA, elfA, csgA, csgD* (curli regulator), or *bcsA* (cellulose). All of the strains were used to infect spinach leaves as described in the text. Serial dilutions of homogenized samples were plated out onto MacConkey-Sorbitol plates for bacterial counting. These data represent the average of three experiments repeated on different days in triplicate. $^*P > 0.05$ statistically significant with respect to the wild-type strain.

sought to determine if the intimin–Tir complex was important for spinach colonization. This analysis showed that intimin (*eae*) and Tir (*tir*) mutants were not significantly abrogated in adherence suggesting that the intimin–Tir interaction is not crucial or necessary for leaf colonization, as it is for human cells. Since the EspP cytotoxin was suggested to play a role in bovine colonization and forms adhesive macrostructures (Dziva et al., 2007; Xicohtencatl-Cortes et al., 2010), we included this mutant in our analysis. The *espP* mutant was not significantly affected in leaf colonization (**Figure 2A**).

ROLE OF QUORUM SENSING IN LEAF COLONIZATION

Quorum-sensing regulates many virulence-associated functions in bacteria such as flagellation, motility, and T3S (Sperandio et al., 2001). We found that a quorum-sensing mutant (Δ*qseB*) was reduced in adherence by 80% as compared to the wild-type strain (**Figure 2A**) and this result correlates with the observation that the flagella (*fliC*) and T3SS (*escN*) mutants were significantly hampered (72 and 52% reduction, respectively) in spinach colonization.

STEC PILI TYPES INVOLVED IN THE COLONIZATION OF BABY SPINACH LEAVES

The specific adherence molecular mechanisms that mediate phyllosphere colonization by STEC remain elusive. Hence, we investigated the role of recently described surface factors of STEC in adherence to spinach leaves. To this aim, isogenic strains carrying mutations in genes encoding for HCP, ECP, ELF, curli, and cellulose were tested for leaf adherence in comparative quantitative experiments. We found that the *csgA* and *csgD* (99% reduction), *ecpA* (83% reduction), and *hcpA* (49% reduction) mutants were significantly hampered in adherence to baby spinach leaves in comparison to

the parental strain EDL933 (**Figures 2B,C**). Notably, when the *csgA* and *csgD* mutants were complemented with plasmids (pCsgA and pCP994 respectively) that over express curli (Saldana et al., 2009), the resulting strains attached more than the wild-type strain to the spinach leaves (**Figure 2C**). The remaining ELF and cellulose mutants were not deficient in spinach colonization (**Figure 2C**). The presence of ECP and curli on the bacteria colonizing the leaf epidermis was demonstrated by IFM using specific antibodies against these pili structures (**Figure 3**). Mutants in curli and ECP served as negative controls for production of these pili. In all, the IFM data compellingly indicate that flagella, ECP, and curli are present during the interaction of the colonizing bacteria with the spinach leaves and participate collectively in adherence and colonization.

LOCALIZATION OF STEC WITHIN LEAF TISSUES

Previous studies have shown STEC bacteria on leaf surfaces, on and around stomata, and between cellular spaces and xylem (Itoh et al., 1998; Solomon et al., 2002; Warriner et al., 2003; Franz et al., 2007). We sought to further investigate the ability of STEC to reside within the stomata internal cavity and internal tissues. SEM analysis of spinach leaves incubated from 1 to 24 h with EDL933 revealed the presence of increasing number of bacteria on the stomata and within its internal cavity (**Figures 4A–F**). Scores of bacteria tethered by filamentous appendages resembling flagella were noted (**Figure 4B**). Accompanying IFM experiments using anti-O157 LPS antibody showed compelling localization of STEC O157 bacteria on stomata and internalized within stomata cavities (**Figures 4G–J**). Further, analysis of cross sections of infected leaves confirmed

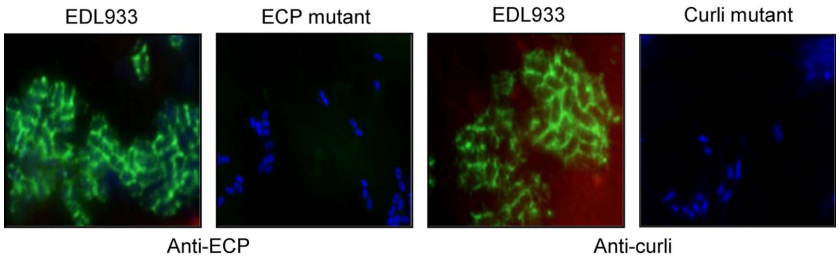

FIGURE 3 | Demonstration of ECP and curli on adhering bacteria. Leaves infected with wild-type strain, ECP or curli mutants (bacteria in blue) were immunostained with specific antibodies against ECP and curli (pili in green). Note the presence of these pili only on EDL933.

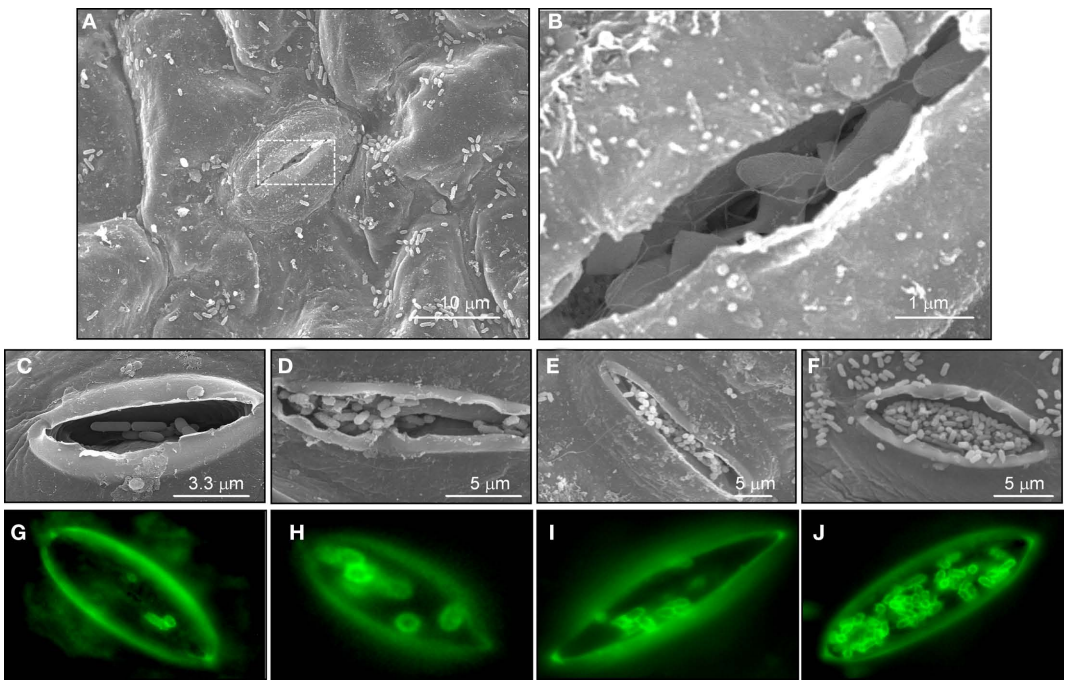

FIGURE 4 | Evidence of STEC in stomata. (A) Scanning electron micrograph showing bacteria on leaf epidermis at 6 h of infection. **(B)** High magnification of boxed area in **(A)** showing flagellate bacteria internalized in the stomata. **(C–F)** Micrographs (60X) of time-course EDL933 infection experiments between 3, 6, 12, and 24 h showing progressive association of bacteria with stomata. **(G–J)** Same experiment as before employing IFM and anti-0157 antibodies to stain bacteria (green).

the presence of STEC bacteria within stomata internal cavities, intercellular spaces of the spongy mesophyll, and notably in the vascular tissue (xylem and phloem; **Figure 5**).

Based on these observations, we hypothesized a role for T3SS in leaf internalization. Thus, we then compared the ability of EDL933 versus the *escN* mutant to penetrate the leaf surface using the gentamicin protection assay. We found that EDL933 survived at a rate of 8.3% after gentamicin treatment, while the *escN* mutant showed 6.1% survival (data not shown). A more clear involvement of T3SS was seen when *E. coli* K-12 DH5α and DH5α carrying the LEE region, which encodes the T3SS on a plasmid (pLEE), were included in the assay. Leaves infected with DH5α (pLEE) showed 3.2-fold-increase with respect to the DH5α strain (data not shown). Other workers have shown STEC and enteroaggregative *E. coli* adhering to the leaf epidermis, and on and around stomata but not beyond the substomal chamber (Berger et al., 2009, 2010; Xicohtencatl-Cortes et al., 2009). Based on previous observations and our own, we hypothesized that *E. coli* O157:H7 are able to provide for themselves with a safe, nutrient-rich niche in the womb of stomata, internal tissues, and intercellular spaces of the leaf, where they are able to replicate and survive, protected from environmental foes.

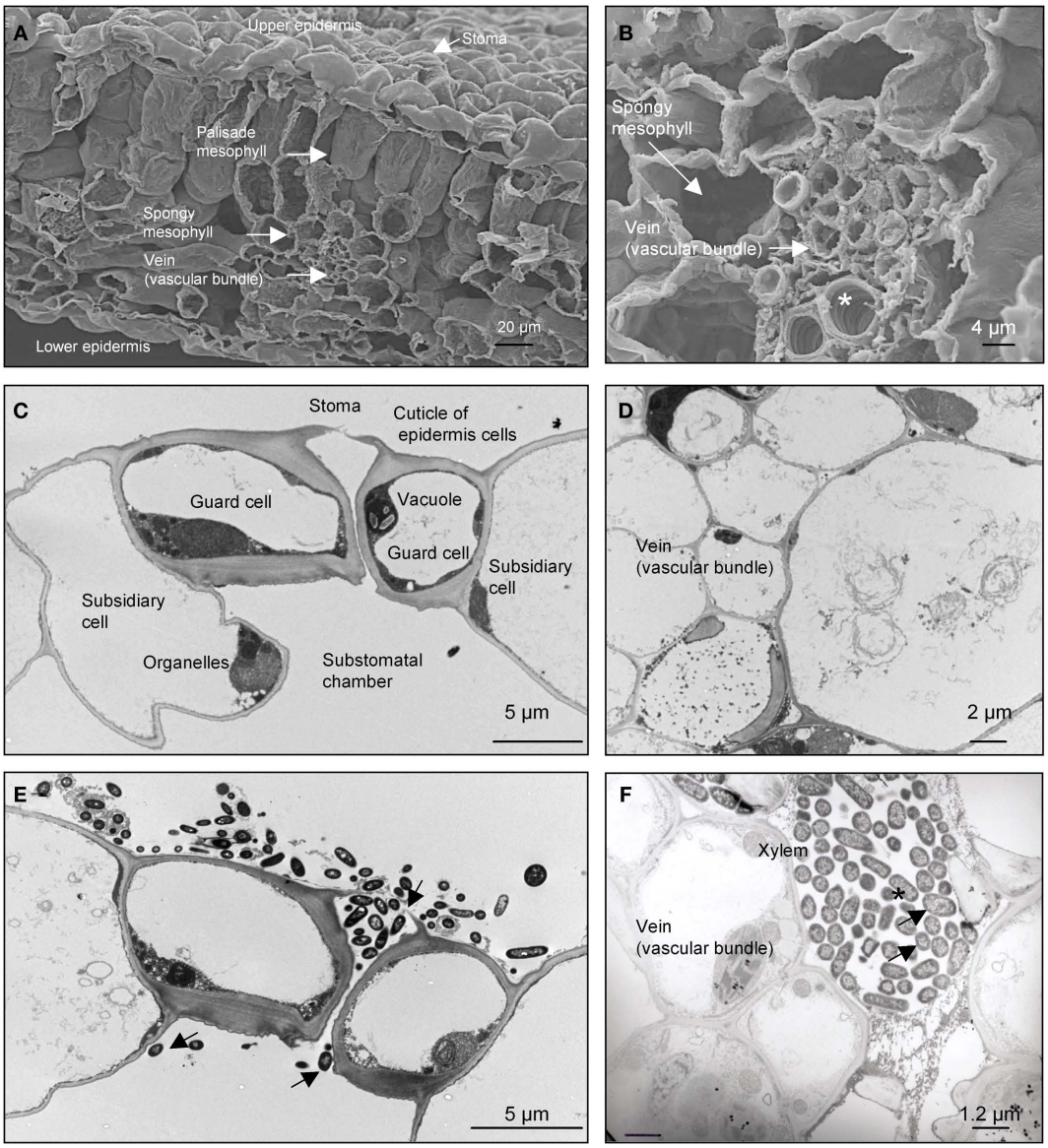

FIGURE 5 | Evidence of STEC present in stomata chambers and in leaf vein. (A) SEM image showing a cross section of uninfected leaf. **(B)** Close up of the internal tissue of the leaf showing the vascular bundle and spongy mesophyll structures. **(C,D)** TEM images showing cross sections of uninfected leaf after 24 h in DMEM. Anatomical sites are indicated with arrows. **(E)** Cross section of leaf infected with EDL933 (arrows) for 24 h revealing colonization on the cuticle of the epidermis, stomata, and the substomatal chamber. **(F)** Cross section showing internal structures of the spinach leaf and colonization of the vessel elements of the xylem with STEC (arrows).

LEAF INTERNALIZATION PROVIDES PROTECTION AGAINST BACTERICIDAL AGENTS

Is stomata colonization a "hide-n-sick" mechanism by which STEC evades industrial decontamination of agricultural products for human consumption? In an attempt to address this important question we subjected experimentally STEC-tainted spinach leaves to decontamination treatments with 0.1–10% of hypochlorite solutions and also to exposure to ozonated water. First, we showed that 10% sodium hypochlorite (equivalent to 6,500 ppm) and 125 ppm of ozonated water killed 100 and 99.9% of the bacteria, respectively (**Figures 6B,D**). Second, we subjected STEC-infected leaves to the same treatments after which bacteria were still recovered (**Figures 6A,C**). Although these anti-bacterial agents reduced the bacterial population on the leaves by ~50% as compared to the mock control, it is clear that a striking number of bacteria residing in the stomata resisted and survived these bactericidal treatments. A reasonable explanation for these results is that internalization of bacteria into stomata and internal compartments of the leaf protected bacteria from bactericidal agents such as antibiotics (e.g., gentamicin), chlorine or ozone.

DISCUSSION

The present study was initiated to address important questions concerning the interaction of STEC with spinach leaves. Specifically, we wanted to identify which of the adhesive factors of STEC, known to be important for human and animal intestinal epithelial cell infection, are also engaged in colonization and survival of these bacteria in baby *Spinacia oleracea* leaves. Several lines of evidence, obtained through different experimental approaches support the notion of the participation of flagella in baby spinach leaf colonization. We demonstrated in time-course experiments assisted by high-resolution SEM and IFM that the bacteria multiply and colonize the leaf surface and that flagella are produced progressively. In support of these observations, we found that an EDL933 *fliC* (flagella) isogenic mutant exhibited significant decreased attachment to spinach leaves. In line with our data, Carey et al. (2009) found that *fliC* was up-regulated during storage of Romaine lettuce at 15°C. It is apparent the flagella display other functions than motility.

A unifying theme in animal and plant bacterial pathogens is the production of specialized secretion mechanisms for direct delivery of effector proteins into host cells to inflict damage. Plant pathogens such as *Pseudomonas* and *Agrobacterium* employ flagella, pili, T3SS or T4SS to infect and cause phytopathology, although the nature of their receptors remains unknown (Korhonen et al., 1986; Rahme et al., 1995; McCullen and Binns, 2006). Flagellation and T3S of STEC are regulated by a double component (*qseBC*) quorum-sensing system (Sperandio et al., 2001). We found that STEC *escN* (T3SS-ATPase) and *qseB* mutants were significantly impaired in leaf

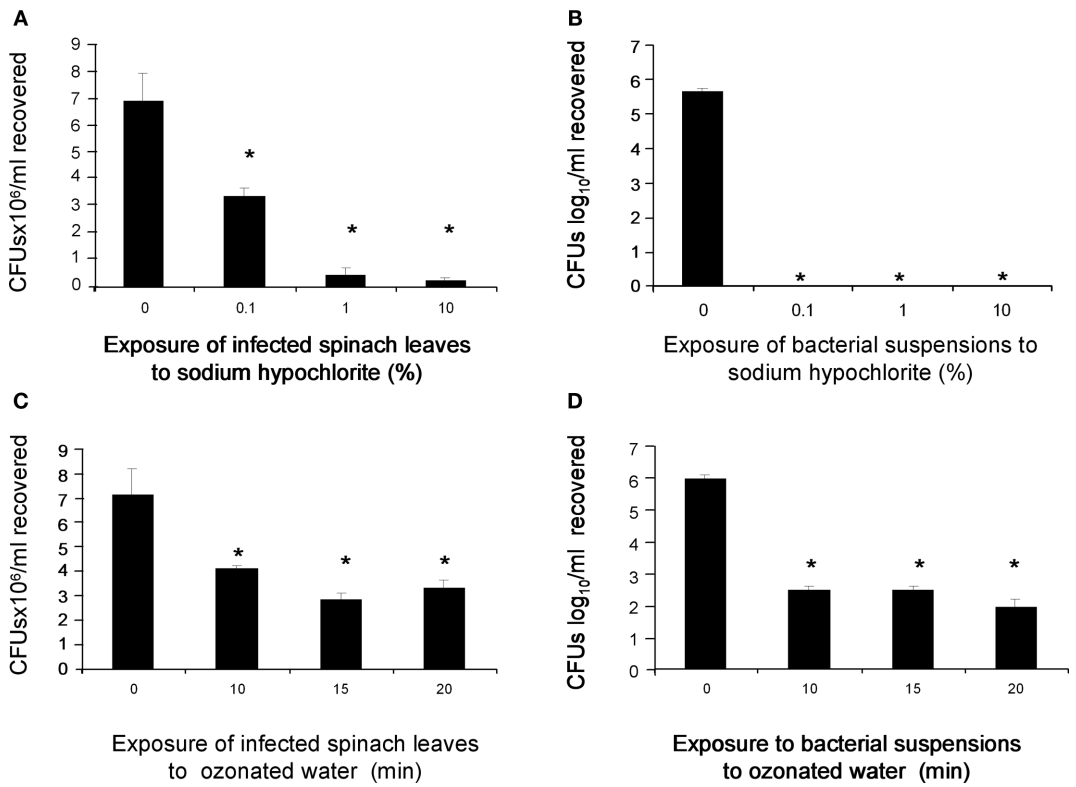

FIGURE 6 | Survival of STEC within stomata to different decontamination processes. (A) STEC infected-spinach leaves for 9 h were treated for 10 min with 0–10% chlorine solutions and then plated out onto MacConkey-Sorbitol agar. Note that even at 10% of chlorine wash, the bacteria were able to grow suggesting that they are protected within the stomata. **(B)** A suspension of 10^6 bacteria was treated for 10 min with 0–10% chlorine solutions. **(C)** Treatment of infected leaves for 9 h with and without ozonated water. **(D)** A suspension of 10^6 bacteria was treated with and without ozonated water from 0 to 20 min. These data represent the average of three experiments repeated on different days in triplicate. *$P > 0.05$ statistically significant.

colonization with respect to the parental strain, indicating that the T3SS and cell-to-cell communication are important in the interaction of STEC with plant cells. While the intimin–Tir complex is crucial for attachment and the characteristic development of actin-rich processes on host cells, neither intimin nor Tir seemed to be required for leaf adherence. This was somewhat expected given the striking differences in chemical composition between human and plant tissue surfaces.

We sought to investigate if pili and cellulose were required for leaf colonization. Comparative adherence studies employing isogenic pili mutants revealed that curli, ECP, and HCP were required for spinach leaf adherence while ELF and cellulose were apparently not. It is well established that pili, in particular curli, are highly hydrophobic appendages that neutralize negative charges on host cells facilitating adherence (Collinson et al., 1991). Recently, it was shown that the level of adherence of *E. coli* O157:H7 strains to cabbage and iceberg lettuce surfaces correlated with the amount of curli produced and their hydrophobicity (Patel et al., 2011). The demonstration that several redundant adhesive factors are required for spinach leaf colonization provides an immediate conceptual framework to explain the multiple molecular mechanisms of plant infection by a human pathogen. Future studies will be needed to elucidate the nature of the plant receptors involved in the recognition of STEC fimbrial adhesins.

Plant pathogens are able to breach into internal tissues using wounds and natural openings of the phyllosphere (Hirano and Upper, 2000; Melotto et al., 2006), but the molecular mechanisms employed to penetrate through stomata, as a natural gateway for passage into internal tissues and cause phytopathology are unknown. Compelling data from ultrastructural, genetic, and functional studies described here clearly showed the presence of STEC O157 within stomata and electron microscopy analysis of leaf cross sections revealed the presence of aggregates of bacteria in internal compartments of the leaf such stomata chambers, intercellular spaces, xylem (specifically in vessel elements), and phloem. Experiments designed to evaluate the ability of STEC to survive within spinach leaves upon treatment with sodium hypochlorite solutions and ozonated water showed that live bacteria could be recovered suggesting that the internal spaces of spinach leaves protect bacteria against these bactericidal agents. We showed that the T3SS is involved in internalization and survival of STEC in spinach leaves. Transformation of a non-pathogenic *E. coli* with pLEE (T3SS+) rendered the bacteria capable of adhering to the epidermis, internalizing into leaf tissue allowing themselves to survive the bactericidal treatments. It is possible that STEC possess mechanisms that direct them toward stomata (e.g., through chemotaxis, motility, quorum sensing) and induce guard cells to open these gateways. The low-infectious dose of STEC (10–50 organisms) and its ability to colonize the stoma and internal leaf compartments provide logical explanations by which the bacteria survive man-driven decontamination treatments and are able to cause outbreaks of STEC infections associated with consumption of tainted-agricultural products. Thus, infected leafy greens may represent potential vehicles of infection and transmission to humans. In conclusion, our research shows that STEC, a human pathogen, which uses several pili types, flagella and the T3SS to colonize human intestinal tissue, can also employ these mechanisms to gain access to plant leaf surface, internal tissues and intercellular spaces where the bacteria survive in a protected microenvironment before causing disease.

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Characterization of EhaJ, a new autotransporter protein from enterohemorrhagic and enteropathogenic *Escherichia coli*

Donna M. Easton[1,2], Makrina Totsika[2], Luke P. Allsopp[2], Minh-Duy Phan[2], Adi Idris[2], Daniël J. Wurpel[2], Orla Sherlock[2†], Bing Zhang[3], Carola Venturini[2], Scott A. Beatson[2], Timothy J. Mahony[3,4], Rowland N. Cobbold[1] and Mark A. Schembri[2]*

[1] School of Veterinary Science, The University of Queensland, Gatton, QLD, Australia
[2] Australian Infectious Disease Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD, Australia
[3] Animal Science, Agri-Science Queensland, Department of Employment, Economic Development and Innovation, Brisbane, QLD, Australia
[4] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD, Australia

Edited by:
Elizabeth L. Hartland, The University of Melbourne, Australia

Reviewed by:
Toru Tobe, Osaka University, Japan
Ian Henderson, University of Birmingham, UK

***Correspondence:**
Mark A. Schembri, School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia, QLD 4072, Australia.
e-mail: m.schembri@uq.edu.au

†Present address:
Orla Sherlock, School of Health and Science, Department of Applied Sciences, Dundalk Institute of Technology, Dundalk, Ireland.

Enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are diarrheagenic pathotypes of *E. coli* that cause gastrointestinal disease with the potential for life-threatening sequelae. While certain EHEC and EPEC virulence mechanisms have been extensively studied, the factors that mediate host colonization remain to be properly defined. Previously, we identified four genes (*ehaA*, *ehaB*, *ehaC*, and *ehaD*) from the prototypic EHEC strain EDL933 that encode for proteins that belong to the autotransporter (AT) family. Here we have examined the prevalence of these genes, as well as several other AT-encoding genes, in a collection of EHEC and EPEC strains. We show that the complement of AT-encoding genes in EHEC and EPEC strains is variable, with some AT-encoding genes being highly prevalent. One previously uncharacterized AT-encoding gene, which we have termed *ehaJ*, was identified in 12/44 (27%) of EHEC and 2/20 (10%) of EPEC strains. The *ehaJ* gene lies immediately adjacent to a gene encoding a putative glycosyltransferase (referred to as *egtA*). Western blot analysis using an EhaJ-specific antibody indicated that EhaJ is glycosylated by EgtA. Expression of EhaJ in a recombinant *E. coli* strain, revealed EhaJ is located at the cell surface and in the presence of the *egtA* glycosyltransferase gene mediates strong biofilm formation in microtiter plate and flow cell assays. EhaJ also mediated adherence to a range of extracellular matrix proteins, however this occurred independent of glycosylation. We also demonstrate that EhaJ is expressed in a wild-type EPEC strain following *in vitro* growth. However, deletion of *ehaJ* did not significantly alter its adherence or biofilm properties. In summary, EhaJ is a new glycosylated AT protein from EPEC and EHEC. Further studies are required to elucidate the function of EhaJ in colonization and virulence.

Keywords: autotransporters, EHEC, EPEC, adhesin, biofilm

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) are pathotypes of *E. coli* responsible for different pathologies in humans. EPEC are associated with small intestinal enteritis, and are a major cause of childhood diarrhea (Nataro and Kaper, 1998). STEC may also be associated with diarrhea, with some strains inducing more severe forms of enteritis such as hemorrhagic colitis, or extraintestinal disease such as hemolytic uremic syndrome (Karch et al., 2005). Such enhanced virulence STEC strains are referred to as enterohemorrhagic *E. coli* (EHEC). Both pathogens and their associated diseases are prevalent globally, with EPEC being a more significant cause of morbidity and mortality in developing countries (Nataro and Kaper, 1998; Bardiau et al., 2010). EPEC are generally considered to be communicable pathogens, being transmitted from human to human via the fecal-oral route. STEC (and therefore EHEC) are recognized zoonotic pathogens, with ruminant livestock being the principal host (Nataro and Kaper, 1998; Gyles, 2007). Food-borne transmission is important in the epidemiology of both EPEC and EHEC.

The molecular mechanisms associated with the colonization of human and animal hosts by EHEC and EPEC are not fully understood. The locus for enterocyte effacement (LEE) encodes a type three secretion system that is found in representative strains of both EHEC and EPEC. Whilst there are component protein and tissue tropism differences between LEE products (particularly for intimin, the key effector) for EPEC and EHEC, this mechanism appears functionally analogous in both pathogens in contributing to host cell attachment (Bardiau et al., 2010). In EHEC, several additional adhesins including Iha (Tarr et al., 2000), long polar fimbriae (Torres et al., 2002), curli (Uhlich et al., 2001), F9 fimbriae (Dziva et al., 2004; Low et al., 2006), Saa (Paton et al., 2001), and Efa1 (Nicholls et al., 2000) have been described. The roles and mechanisms of these adhesins (individually and/or in concert)

in mediating host colonization remain to be fully elucidated. An improved understanding of EPEC and EHEC mucosal adherence may lead to development of interventions that will disrupt host colonization, be it colonization of humans as a prelude to pathogenesis, or colonization of livestock leading to carriage and maintenance of EHEC that can be subsequently transmitted to humans.

Several cell-surface proteins from the type V secreted autotransporter (AT) class have been characterized from EHEC. AT proteins are common to many Gram-negative pathogens and have diverse functions ranging from cell-associated adhesins to secreted toxins. All AT proteins have several common features: an N-terminal signal sequence, a passenger (α) domain that often encodes a virulence function and is either anchored to the cell surface or released into the external milieu and a translocation (β) domain that resides in the outer membrane (Jose et al., 1995; Henderson et al., 1998). Three broad categories of AT proteins have been defined in the literature based on domain-architecture: the serine protease AT proteins of *Enterobacteriaceae* (SPATEs), the AIDA-I type AT proteins and the trimeric AT adhesins (TAAs; Henderson et al., 2004). Among the AIDA-I group, the Ag43 protein (found in most *E. coli* strains), the TibA adhesin (associated with some enterotoxigenic *E. coli*) and the AIDA adhesin (associated with some diarrhea-causing *E. coli*) represent well characterized AT proteins that mediate aggregation, biofilm formation and can be glycosylated (Klemm et al., 2006). We previously described the identification and characterization of four AT proteins from *E. coli* EDL933 that belong to the AIDA-I group (Wells et al., 2008, 2009). Here we have extended this analysis to include a larger collection of EHEC and EPEC strains and also examined the prevalence of seven recently identified AT-encoding genes, two from the AIDA-I group (i.e., groups 6 and 7) and five from the TAA group (i.e., groups 1–5; Wells et al., 2010). In this study we have examined the relative prevalence of the various identified AT genes among EPEC and EHEC strains. We have also characterized the functional properties of a newly recognized AT, namely EhaJ.

MATERIALS AND METHODS

STRAINS AND MEDIA

The EHEC and EPEC strains used to assess the prevalence of AT-encoding genes were obtained from CSIRO Food and Nutritional Sciences, Queensland Health Forensic and Scientific Services, and the New South Wales Department of Primary Industries. EPEC MS455 is an O27:H6 strain from our laboratory collection and was positive for *ehaA*, *ehaB*, *ehaC*, *ehaJ*, and a group 4/5 AT-encoding gene by PCR screening. *E. coli* MS427 (MG1655*flu*) and OS56 [green fluorescent protein (GFP)-tagged MG1655*flu*] have been described previously (Kjaergaard et al., 2000; Sherlock et al., 2004). Cells were grown at 37°C on solid or in liquid Luria–Bertani (LB) media supplemented with the appropriate antibiotics unless otherwise stated. Where necessary, gene expression was induced with 0.2% arabinose.

DNA MANIPULATIONS AND GENETIC TECHNIQUES

Genomic DNA was extracted from overnight cultures using the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's procedure for Gram-negative bacteria. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. Restriction digests, ligations, and T4 polymerase treatment followed the manufacturer's recommendations (New England BioLabs). PCR reactions for which high fidelity was required were performed using Expand High Fidelity Polymerase System (Roche) following the manufacturer's recommendations. *Taq* DNA polymerase (New England BioLabs) was used for screening PCR reactions. DNA sequencing was performed by the Australian Equine Genetics Research Centre.

PREVALENCE OF AT-ENCODING GENES

The prevalence of each AT-encoding gene was assessed by PCR using primers specific to the translocation-encoding domain. Primers for *ehaA*, *ehaB*, *ehaC*, and *ehaD* were as previously described (Wells et al., 2008). Primers for the group 1, 2/3, 4/5, and 6/7 AT-encoding genes were as follows: group 1: 2105 (5′-gggtatggctctcaggtgaa) and 2106 (5′-agcatcagcaacagcatcac); group 2/3: 2107 (5′-acgyctgracagccagcagc) and 2108 (5′-gcggtctgctcgttgaagcg); group 4/5: 2109 (5′-caaatkcarmgtctggcgca) and 2110 (5′-cagacacccgagattcaccg); group 6/7: 2111 (5′-tgccayhtggtttgccgatg) and 2112 (5′-agayrrcctgtgcctgtggc). Prevalence of the *ehaJ* gene (which belongs to group 6) was assessed using primers 2442 (5′-aaggcggggaatgcagcgtc) and 2443 (5′-gcgtcaggctgagcgtgtgt). The groups were combined for initial screening where sufficient sequence conservation existed.

CONSTRUCTION OF PLASMIDS

The *egtA–ehaJ* and *ehaJ* genes were amplified by PCR from MS455 using primers designed from the available genome sequence of EPEC strain 2348/69. The following primers were used: *egtA–ehaJ* (141, 5′-cgcgctcgagataataaggagctttacagtgagtaataat and 142, 5′-cggcgaagcttctctgtattttaccaactgt); *ehaJ* (143, 5′-cgcgctcgagataataaggactaattcatgaacagaactt and 142). The PCR products containing *egtA–ehaJ* and *ehaJ*, respectively, were digested with *Xho*I (forward primer) and *Hin*dIII (reverse primer) and ligated to *Xho*I–*Hin*dIII digested plasmid pBAD/*Myc*-HisA to generate plasmids pOMS2 (contains *egtA–ehaJ*) and pOMS3 (contains *ehaJ*). In both constructs, expression of *ehaJ* is under control of the arabinose-inducible *araBAD* promoter (Guzman et al., 1995) and a stop codon is included so as to produce the recombinant protein without any tags introduced by the vector. The kanamycin cassette from pUC4K (GE Healthcare Life Sciences) was subsequently inserted into the *Hin*dIII site of pOMS2 to generate pOMS2-kan and enable its transformation into *E. coli* OS56. The pBAD/*Myc*-HisA vector was similarly manipulated to produce the negative control plasmid pBAD-kan.

CONSTRUCTION OF AN *ehaJ* MUTANT IN EPEC STRAIN MS455

The *ehaJ* gene was deleted from *E. coli* MS455 using a modification of the λRed recombinase gene replacement system (Datsenko and Wanner, 2000). The kanamycin cassette was amplified from pKD4 using primers 2571 (5′-ggcggatccgtgtaggctggagctgcttc) and 2572 (5′-ggcggatcccatatgaatatcctccttag), which include *Bam*HI recognition sites. Flanking DNA consisting of approximately 500 bp up- and down-stream of *ehaJ* were amplified using primer pairs 2562 (5′-gccgctacagcaacgggtgga)/2575 (5′-ggcggatccgatgtggattccgcctgcgc) and 2576 (5′-ggcggatccacacactgaccatcaacggc)/2565 (5′-cgcatccagacactgccatct). The PCR products

were digested with *Bam*HI and the flanking regions ligated with T4 DNA ligase, PCR amplified, A-tailed and inserted in to the pGEM-T easy vector (Promega). The resulting plasmid was again digested with *Bam*HI and the kanamycin cassette was ligated in between the two flanking regions. The ∼2.5 kb construct was then amplified using the outer most primers (2562 and 2565). This construct was used to transform MS455(pKD46) by electroporation; transformants were selected by growth on agar containing kanamycin (50 μg/mL) and screened by PCR using primers 2599 (5′-gagcagatattctgcgaata), 2600 (5′-ttgagctttcaggctcgcc), 2601 (5′-gctaccgagtgctgtgcatc), and 2602 (5′-tgtcggtgtcggcattgacg) in combination with primers specific for the kanamycin cassette (1287, 5′-ttgcacgcaggttctccg and 1288, 5′-acagctgcgcaaggaacg). The correct insertion of the kanamycin cassette was confirmed by sequencing using these same primers. This strain was referred to as MS455*ehaJ*.

ANTISERUM PRODUCTION AND IMMUNOBLOTTING

A 6× histidine-tagged truncated form of EhaJ was constructed. Primers 2377 (5′-tacttccaatccaatgcggaatccacatctgaggtgacg) and 2378 (5′-ttatccacttccaatgttaccaggacagtgaagtggtcag) were used to amplify the predicted passenger-encoding domain, which was then inserted into the pMCSG7 vector by ligation-independent cloning and maintained in *E. coli* DH5α. This plasmid was then transferred to *E. coli* BL21 for expression of the recombinant protein by induction with 1 mM IPTG and purification by Ni-NTA Superflow columns (Qiagen) under denaturing conditions. Protein purity was assessed by SDS-PAGE analysis as previously described (Ulett et al., 2006). Polyclonal anti-EhaJ serum was raised in rabbits by the Institute of Medical and Veterinary Sciences (South Australia). For immunoblotting, cell lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride microporous membrane filters as described previously (Ulett et al., 2006). Serum raised against the passenger subunit of EhaJ was used as primary serum and the secondary antibody was alkaline phosphatase-conjugated anti-rabbit immunoglobulin G; 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetraolium (BCIP/NBT) was used as the substrate in the detection process. Glycosylation of EhaJ was demonstrated by staining of SDS-PAGE separated proteins from whole cell lysates of MS427(pOMS2) with the Glycoprofile III stain (Sigma-Aldrich) according to the manufacturer's recommendations.

ANALYSIS OF BIOFILM FORMATION

Biofilm formation on polystyrene surfaces was assessed in 96-well microtiter plates (Iwaki) essentially as previously described (Kjaergaard et al., 2000). Briefly, MS427(pOMS2) or MS427(pOMS3) and MS427(pBAD) were grown for 24 h in glucose M9 minimal medium (containing 0.2% arabinose for induction of gene expression) at 37°C, washed to remove unbound cells and stained with crystal violet. Quantification of bound cells was assessed by adding acetone–ethanol (20:80) to dissolve the crystal violet, and the optical density was measured at 595 nm.

Flow chamber experiments were performed essentially as previously described (Allsopp et al., 2010) in M9 minimal media. Briefly, GFP-tagged strains OS56(pBAD-kan) and OS56(pOMS2-kan) were allowed to form biofilms on glass surfaces in a multi-channel flow system that permitted *in situ* monitoring of community structures. Flow cells were inoculated with OD_{600} standardized cultures grown overnight in M9 medium containing kanamycin. Biofilms were analyzed over 48 h, with confocal images captured at 16 and 48 h.

MICROSCOPY AND IMAGE ANALYSIS

An anti-EhaJ-specific serum was used for immunofluorescence microscopy as previously described (Wells et al., 2008). Briefly, overnight cultures of MS427(pBAD), MS427(pOMS2), and MS427(pOMS3) induced with 0.2% arabinose were fixed and incubated with primary rabbit polyclonal anti-EhaJ serum followed by incubation with a secondary goat anti-rabbit IgG antibody conjugated to FITC. Microscopic observation of biofilms and image acquisition were performed on a scanning confocal laser microscope (LSM510 META, Zeiss) equipped with detectors and filters for monitoring of GFP. Vertical cross sections through the biofilms were visualized by using the Zeiss LSM image examiner. Images were further processed for display by using Photoshop software (Adobe, Mountain View, CA, USA). For analysis of the flow cell biofilms, z-stacks were analyzed using COMSTAT software program (Heydorn et al., 2000).

ECM PROTEIN BINDING ASSAYS

Bacterial binding to MaxGel Human ECM (Sigma-Aldrich) and extra cellular matrix (ECM) proteins was performed in a microtiter plate ELISA assay. Microtiter plates (MaxiSorp; Nunc) were coated overnight at 4°C with 2 μg of MaxGel Human ECM or individually with the following ECM proteins (final amount of 2 μg/well): collagen type I, type II, type III, type IV, and type V, fibronectin, fibrinogen, laminin, elastin, heparin sulfate, and BSA (Sigma-Aldrich). Wells were washed twice with TBS (137 mM NaCl, 10 mM Tris pH 7.4) and then blocked with TBS-2% skim milk for 1 h. After being washed with TBS, 200 μL of bacterial cell suspension (standardized to $OD_{600} = 0.1$) was added and the plates were incubated at 37°C for 2 h. After being washed to remove non-adherent bacteria, adhered cells were fixed with 4% paraformaldehyde, washed, and incubated for 1 h with anti-*E. coli* serum (Meridian Life Sciences Inc., #B65001R) diluted 1:500 in 0.2% skim milk, 0.05% Tween-20 in TBS, washed and incubated for 1 h with a secondary anti-rabbit horseradish peroxidase antibody (Sigma-Aldrich; #A6154) diluted 1:1000 in 0.2% skim milk, 0.05% Tween-20 in TBS. After a final wash adhered bacteria were detected by adding 150 μL of 0.3 mg/ml ABTS [2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] (Sigma-Aldrich) in 0.1 M citric acid pH 4.3, activated with 1 μL/mL 30% hydrogen peroxide, and the absorbance was read at 405 nm. Mean absorbance readings were compared with negative control readings [MS427(pBAD)] using two-sample *t*-tests within the Minitab V14 software package (Coventry, UK). *P* values <0.05 were considered significant.

RESULTS

PREVALENCE OF AT-ENCODING GENES IN EHEC AND EPEC

The prevalence of selected AT-encoding genes from the AIDA-I and TAA groups was assessed by PCR screening of EHEC and EPEC strains from our laboratory collection. For this purpose,

we employed primers designed to amplify a region within the conserved translocation-encoding domain of each gene. We found that a correct sized product was amplified from representative EHEC and EPEC strains for *ehaA*, *ehaB*, *ehaC*, *ehaD*, and the group 2, 3, 4, 5, 6, and 7 AT-encoding genes (**Table 1**). In contrast, no PCR product was obtained for the group 1 AT-encoding genes for any of the strains. Most of the EHEC and EPEC strains returned a positive result for *ehaA*, *ehaB*, and *ehaC*, indicating that these AT-encoding genes are highly prevalent. The group 2/3 PCR product was only amplified from EHEC strains (18%), while the group 4/5 PCR product was only amplified from EPEC strains (19%).

While the initial screening of the group 6/7 AT-encoding genes was based on a conserved set of primers for both groups, further screening with primers specific for the group 6 gene indicated that this gene is present in 12/44 (27%) of EHEC and 2/20 (10%) of EPEC strains. We focused the remainder of our study on this gene, and in accordance with the terminology adopted for other AT-encoding genes of EHEC (Wells et al., 2008), we have termed the group 6 AT-encoding gene *ehaJ*.

Table 1 | Prevalence of AT-encoding genes in EHEC and EPEC.

Gene	EHEC ($n = 44$)	EPEC ($n = 21$[a])	AT type[b]
ehaA	43 (97%)	18 (86%)	AIDA-I
ehaB	41 (93%)	21 (100%)	AIDA-I
ehaC	44 (100%)	21 (100%)	AIDA-I
ehaD	5 (11%)	5 (24%)	AIDA-I
Group 1	0	0	TAA
Group 2/3	8 (18%)	0	TAA
Group 4/5	0	4 (19%)	TAA
Group 6/7	44 (100%)	21 (100%)	AIDA-I
ehaJ	12 (27%)	3 (14%)	AIDA-I

[a] *Including bioinformatic screening of EPEC strain E2348/69.*

[b] *Autotransporter type as indicated by Wells et al. (2010).*

ehaJ LIES ADJACENT TO A PUTATIVE GLYCOSYLTRANSFERASE GENE

The EPEC strain 2348/69 is the only genome sequenced *E. coli* strain in the NCBI database that contains the *ehaJ* gene (locus tag E2348C_2704). Further investigation of the EPEC E2348/69 genome revealed the presence of a gene encoding a putative glycosyltransferase immediately upstream of *ehaJ* (locus tag E2348C_2705), which we have referred to as EhaJ glycosyltransferase or *egtA* (**Figure 1**). This tandem glycosyltransferase-AT gene arrangement is similar to that observed for the AidA and TibA AT proteins (Benz and Schmidt, 1989; Elsinghorst and Kopecko, 1992). EhaJ shares 26.1% amino acid identity with AidA and 22.7% amino acid identity with TibA. Both the AidA and the TibA AT genes are located immediately downstream of a glycosyltransferase encoding gene (**Figure 1**). The *tib* locus also contains two additional genes, *tibD* and *tibB*, which have been suggested to play a role in regulation (Lindenthal and Elsinghorst, 1999). The predicted product of the *egtA* gene shares 63.9% amino acid identity with the TibC glycosyltransferase from ETEC H10407 (Elsinghorst and Weitz, 1994) and 62.1% amino acid identity with the AIDA-associated Aah heptosyltransferase from EPEC 2787 (Benz and Schmidt, 1989, 1992).

THE *ehaJ* GENE ENCODES A PROTEIN THAT LOCALIZES TO THE CELL SURFACE

In order to determine if EhaJ is glycosylated and if glycosylation affects EhaJ function, we constructed two different plasmids. We selected one *ehaJ*-positive EPEC strain from our collection, MS455, and PCR amplified and cloned the *egtA–ehaJ* fragment as a transcriptional fusion downstream of the tightly regulated *araBAD* promoter in pBAD/*Myc*-HisA, resulting in plasmid pOMS2. Similarly, a plasmid containing the *ehaJ* gene alone was constructed by PCR amplification and cloning of the *ehaJ* gene from MS455 into pBAD/*Myc*-HisA, resulting in plasmid pOMS3. Unlike the AidA and Ag43 AT proteins, expression of EhaJ did not mediate cell aggregation following growth of these recombinant strains in LB or M9 broth. Furthermore, we found no evidence to indicate

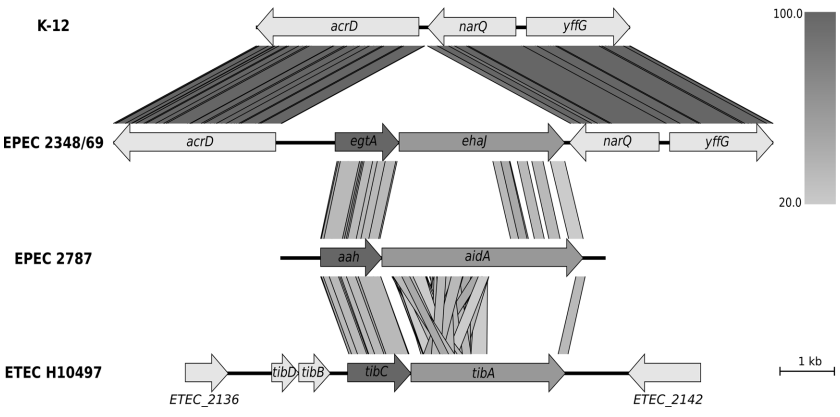

FIGURE 1 | Genomic context of the group 6 autotransporter gene *ehaJ* and its associated glycosyltransferase gene *egtA* in EPEC 2348/69 genome (NC_011601) in comparison with K-12 strain MG1655 (NC_000913), EPEC 2787 (GU810159), and ETEC H10497 (FN649414). Whilst the arrangement of the glycosyltransferase upstream of the autotransporter gene is conserved, the genomic positions of these two genes are different in the four genomes. The figure was generated using Easyfig (http://easyfig.sourceforge.net/; Sullivan et al., 2011) with amino acid sequence comparison (tBLASTx). The level of amino acid identity is shown in the gradient scale.

that the passenger domain of EhaJ could be released from the cell surface following brief heat treatment at 60°C.

EhaJ IS GLYCOSYLATED IN THE PRESENCE OF THE *egtA* GENE PRODUCT

To demonstrate expression of the EhaJ protein, plasmids pOMS2 and pOMS3 were transformed into the previously described *E. coli* K-12 *flu* mutant strain MS427, respectively. *E. coli* MS427 is unable to mediate the classical cell aggregation and biofilm phenotypes associated with Ag43 expression (Reisner et al., 2003). EhaJ cell-surface expression was demonstrated by immunofluorescence microscopy (**Figure 2**) using a rabbit polyclonal antiserum targeting a region in the predicted N-terminal passenger domain of EhaJ. Western blot analysis employing the same EhaJ-specific antiserum detected a 155-kDa protein in whole cell lysates prepared from *E. coli* MS427(pOMS2) following induction with arabinose (**Figure 2**). When the same analysis was performed on *E. coli* MS427(pOMS3), a smaller protein of approximately 110 kDa was detected using the EhaJ-specific serum. The difference in the size of EhaJ in the presence and absence of the *egtA* gene provides evidence to suggest that EhaJ is glycosylated. Glycosylation of EhaJ was demonstrated by staining of SDS-PAGE separated proteins from whole cell lysates of MS427(pOMS2) with the Glycoprofile III stain. Staining of the 155-kDa EhaJ protein was only observed in the presence of EgtA (**Figure 2**). A lower molecular weight band that also stained with Glycoprofile III was also visible on this gel (only in the presence of EgtA); we suggest that this band may represent a partially glycosylated version of EhaJ.

EXPRESSION OF GLYCOSYLATED EhaJ MEDIATES BIOFILM FORMATION

To determine whether the EhaJ protein promotes biofilm formation, we examined the effect of EhaJ over-expression in *E. coli* MS427 in a static biofilm assay in a non-treated polystyrene microtiter plate model after growth in M9 minimal medium (**Figure 3A**). MS427(pOMS2), which produces glycosylated EhaJ, exhibited greatly enhanced biofilm production in comparison to both the negative control strain, MS427(pBAD) and the strain expressing EhaJ without the glycosyltransferase [MS427(pOMS3)]. The ability of glycosylated EhaJ to promote biofilm formation was further assessed by over-expression in *E. coli* OS56 (a GFP-tagged derivative of MS427) in dynamic conditions using the continuous flow chamber model, which permits monitoring of the bacterial distribution within an evolving biofilm at the single cell level due to the combination of GFP-tagged cells and scanning confocal laser microscopy. Glycosylated EhaJ [OS56(pOMS2)] promoted strong biofilm growth under these conditions and produced a structure with a depth of approximately 10 μm (**Figure 3B**).

EhaJ MEDIATES ADHERENCE TO EXTRACELLULAR MATRIX PROTEINS

To study the adhesive properties of EhaJ we examined its ability to mediate binding to various cellular and extracellular targets. Initially, we tested the ability of EhaJ to mediate adherence to HeLa and Caco2 cells, however no binding was observed for *E. coli* OS56(pOMS2-kan; data not shown). Next, we tested for the ability of EhaJ to mediate adherence to MaxGel, a commercially available mixture of ECM components including collagens, laminin,

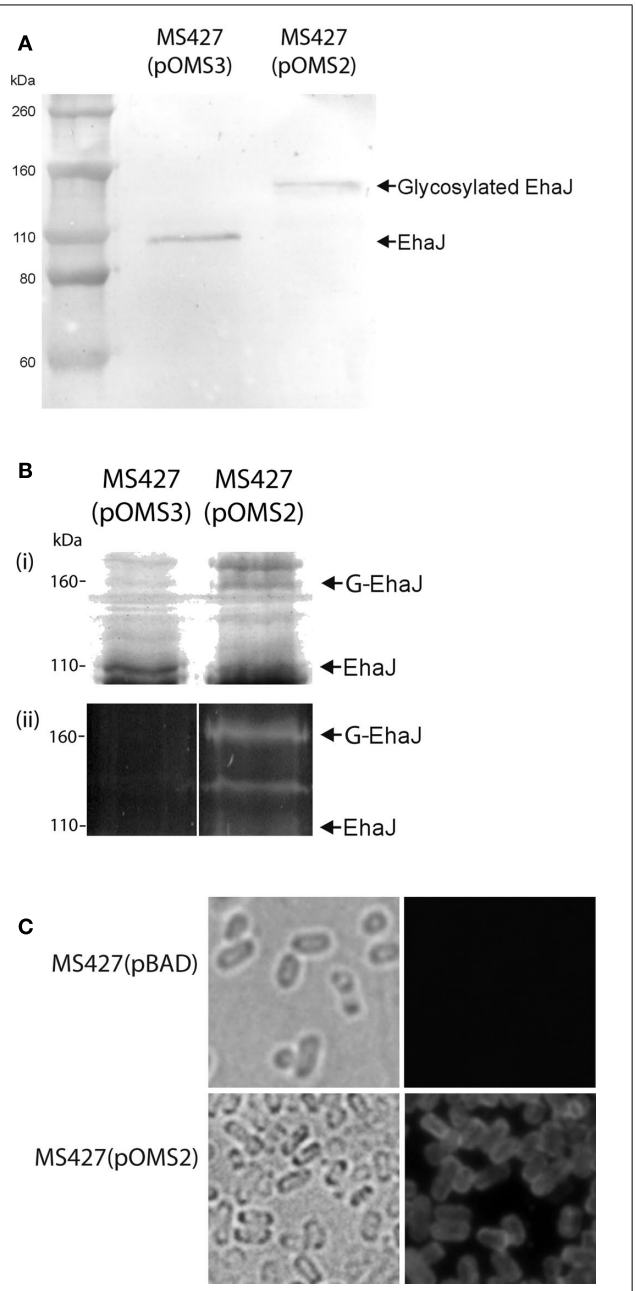

FIGURE 2 | (A) Western blotting with EhaJ-specific antiserum demonstrates the difference in size of EhaJ from MS427(pOMS2) and MS427(pOMS3). Molecular mass markers (M) are pre-stained Novex® Sharp Standard (Invitrogen). **(B)** SDS-PAGE analysis demonstrating **(i)** Coomassie blue staining and **(ii)** Glycoprofile III fluorescent staining of proteins prepared from whole cell lysates of MS427(pOMS2) and MS427(pOMS3). Staining of the 155-kDa EhaJ protein with Glycoprofile III was only observed in the presence of EgtA. A lower molecular weight band that also stained with Glycoprofile III and may represent a partially glycosylated form of EhaJ was also observed in MS427(pOMS2). Although this band is not visible on the western blot shown in **(A)**, it was visible in other western blots that were allowed to develop for a longer time period. **(C)** Immunofluorescence microscopy demonstrating surface localization of EhaJ. Phase-contrast (left) and fluorescence (right) images of MS427(pBAD; top) and MS427(pOMS2; bottom). Strains were grown in the presence of 0.2% arabinose for all three panels. G-EhaJ indicates glycosylated EhaJ.

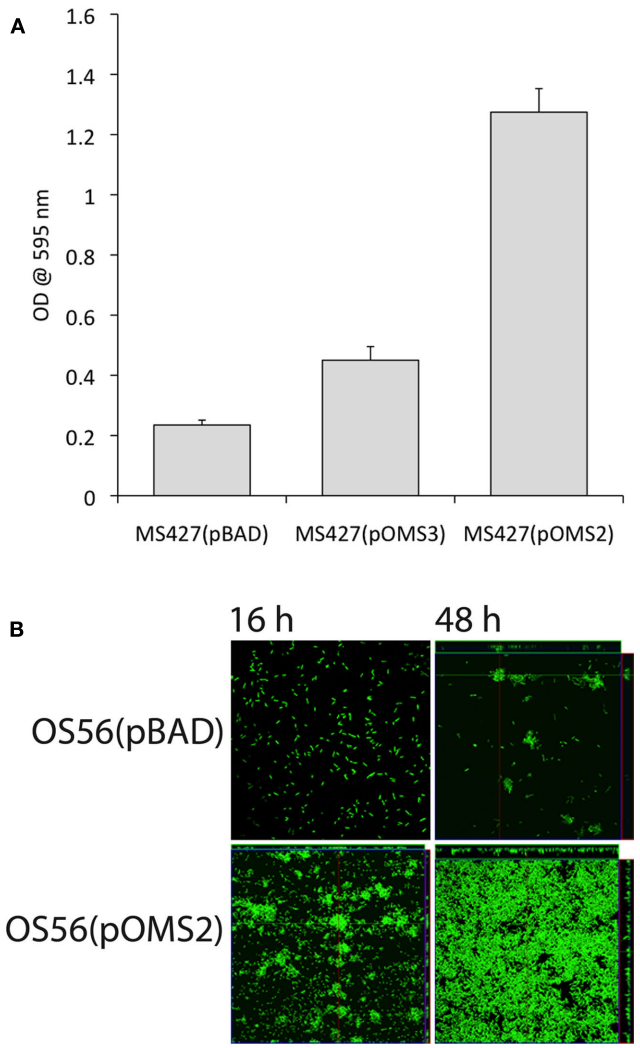

FIGURE 3 | (A) Glycosylated EhaJ [MS427(pOMS2)] mediates enhanced static biofilm formation in a microtiter plate assay at both 28˚C and 37˚C. Results are expressed as the average of six technical replicates within each plate; error bars indicate SD. These results are representative, confirmed by three independent experiments. **(B)** Glycosylated EhaJ mediates enhanced biofilm formation in a dynamic flow cell system at 28˚C. Spatial distribution of dynamic biofilm formation by GFP-labeled *E. coli* strains OS56(pBAD-kan) and *E. coli* OS56(pOMS2-kan) was monitored by confocal scanning laser microscopy at 15 h (left) and 48 h (right) after inoculation. Shown are representative horizontal sections collected within each biofilm and vertical sections (to the right and below for each individual panel) representing the yz-plane and the xz-plane, respectively, at the positions indicated by the white lines.

fibronectin, tenascin, elastin, a number of proteoglycans, and glycosaminoglycans. Both *E. coli* MS427(pOMS2) and *E. coli* MS427(pOMS3) adhered equally well in this assay (**Figure 4**), and thus we examined binding in more detail by testing a range of different ECM proteins. *E. coli* MS427(pOMS2) and *E. coli* MS427(pOMS3) adhered strongly to collagen I, collagen II, collagen III, collagen V, fibronectin, fibrinogen, and laminin (**Figure 4**).

CONSTRUCTION AND CHARACTERIZATION OF AN *ehaJ* MUTANT

To determine whether EhaJ is expressed in wild-type EPEC MS455, we constructed an *ehaJ* deletion mutant employing λ-red mediated homologous recombination of linear DNA (referred to as MS455*ehaJ*). Examination of whole cell lysates prepared from MS455 and MS455*ehaJ*, following growth in LB broth, by SDS-PAGE and western blotting demonstrated a low level of expression of EhaJ in *E. coli* MS455 and an absence of EhaJ in MS455*ehaJ* (**Figure 5**). We then tested MS455 and MS455*ehaJ* for their ability to adhere to MaxGel. MS455 adhered poorly in this assay, and although MS455*ehaJ* adhered less well, the difference was not statistically significant. MS455*ehaJ* complemented with pOMS3 bound strongly to the MaxGel, consistent with results obtained when EhaJ was expressed under control of the *araBAD* promoter in the MS427 background (**Figure 4** and data not shown). Similar results were also obtained with regards to biofilm formation in the microtiter plate assay (data not shown). Thus, while EhaJ is a newly identified AT protein expressed by *E. coli* MS455, further studies will be required to investigate the function of this protein in wild-type strains.

DISCUSSION

Autotransporters are a diverse family of outer membrane proteins that have various virulence-associated functions, including in biofilm formation and adherence to mammalian cells. Our previous study of four AT proteins identified in *E. coli* O157:H7 strain EDL933, termed EhaA, EhaB, EhaC, and EhaD, assessed the prevalence of these genes in STEC strains (Wells et al., 2008). In the present study, we have extended this analysis to include two strain libraries from our collection that have been confirmed by both serotyping and virulence gene analysis as EHEC or EPEC strains, for comparison of prevalence between these two pathotypes. The prevalence of the translocation (β) domain for each of these AT genes in the EHEC collection was found to be similar in this study to that found in our previous study (Wells et al., 2008) and also similar to the prevalence in the EPEC collection (**Table 1**).

Our recent bioinformatic approach to the identification and classification of AT proteins in *E. coli* revealed several predicted AT genes that did not group with those previously characterized and these were termed groups 1–7 (Wells et al., 2010). The present study also sought to assess the prevalence of these newly identified AT genes in our EHEC and EPEC libraries. Groups 1, 2, and 3, which were all closely grouped with the TAA clade, were not detected in our EPEC library in the current study. The group 1 gene was also not detected in the EHEC library and the group 2/3 screen only returned the correct PCR product in 18% of the EHEC strains. Each of these genes was only found in one publically available genome sequence (Wells et al., 2010); the group 1 gene was found in strain SMS 3–5, an environmental strain, and groups 2 and 3 were found in strain ED1a, originally isolated from a healthy adult. Thus it was not unexpected that these apparently rare AT genes would be absent from pathogenic strains. The group 4 gene was originally identified in strain UMN26, a uropathogenic strain, and the group 5 gene was identified in four strains, of differing pathotypes, including EPEC strain E2348/69. The current study indicated a modest prevalence of these genes (approximately 19%) in EPEC strains and an absence in EHEC strains. We note that the

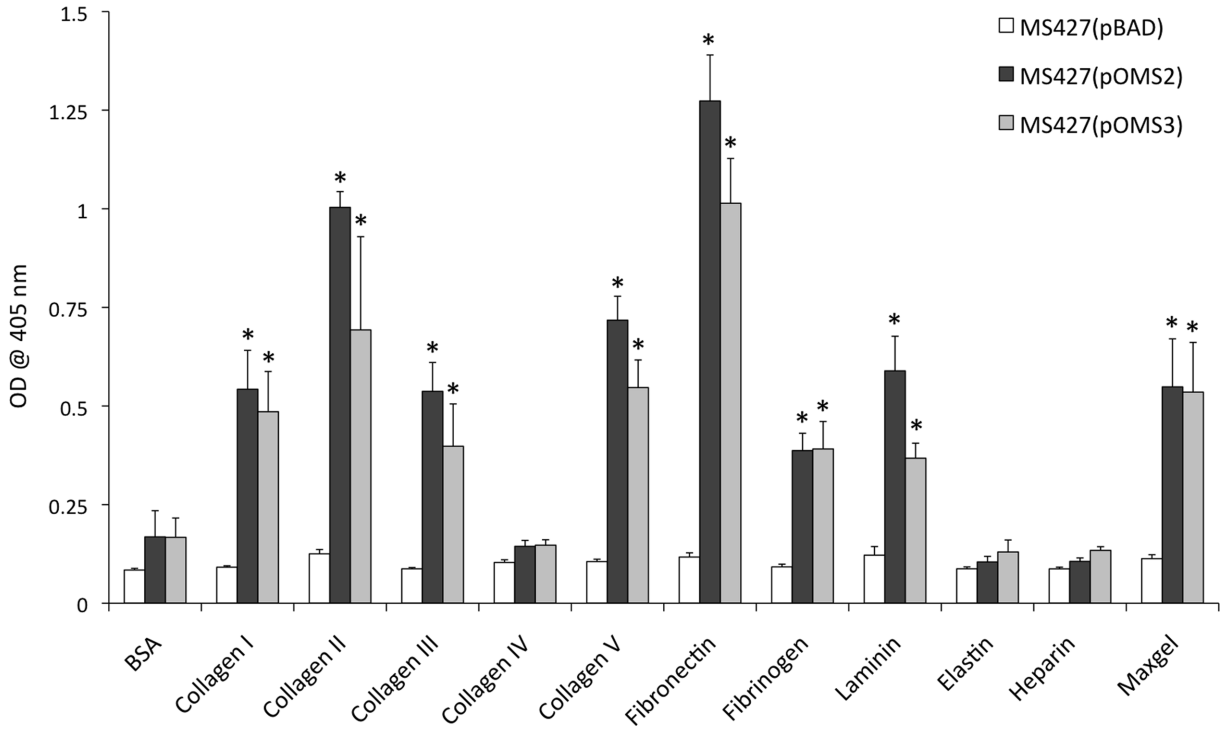

FIGURE 4 | EhaJ mediates attachment to a range of ECM proteins in an ELISA-based binding assay. MS427 bound to MaxGel, collagen I, II, III and V, fibronectin, fibrinogen, and laminin when expressing *ehaJ* with or without co-expression of *egtA* [* indicates significant difference ($P < 0.05$) in comparison with BSA control]. EhaJ did not mediate adherence to collagen IV, elastin, heparin sulfate, or BSA.

PCR-based approach we adopted does not distinguish between the presence of intact genes and pseudogenes. Future studies may address this issue and investigate the presence of these AT genes in different pathotypes of *E. coli*.

Screening for the translocation domain of the group 6 and 7 genes indicated that at least one of these genes is present in all of the strains tested in both the EHEC and EPEC libraries. However, specific screening for the group 6 gene, now termed *ehaJ*, found only a modest prevalence of this gene (15–27%), suggesting that the group 7 gene may be highly prevalent. However, BLASTn and BLASTp searches of *E. coli* genomes available through the NCBI database indicated that the group 7 gene is truncated to only the translocation domain, or parts of the translocation domain, in the majority of strains, explaining why this gene was not identified in more strains in the original study. The group 7 gene was found in only four strains previously, however the current searches identify the full gene in 11 *E. coli* strains and four *Shigella flexneri* strains. We chose to focus on the group 6 AT, which we have termed EhaJ, as analysis of the genomic context of the gene identified in EPEC strain E2348/69 (E2348C_2704) revealed the presence of a predicted glycosyltransferase immediately upstream (E2348C_2705), termed *egtA*. As shown in **Figure 1**, this is similar to the organization of the *aah/aidA* and *tibC/tibA* systems. Comparison of *egtA* with these other AT-associated glycosyltransferases revealed that *egtA* shares approximately 60% identity, with *tibC* and *aah*. Glycosylation of AIDA-I by the *aidA*-associated heptosyltransferase (AAH) is necessary for AIDA-I-mediated adherence to mammalian cells (Benz and Schmidt, 2001; Charbonneau et al., 2007). However, Charbonneau et al. (2007)also demonstrated that glycosylation is not necessary for the cell-binding domain to bind Hep-2 cells, but that glycosylation greatly enhances stability and protease-resistance of AIDA-I. They therefore suggest that glycosylation is needed for adherence only indirectly, in that it likely stabilizes the conformation of the cell-binding domain. TibA was the first glycosylated AT described in *E. coli* and a recent study has shown that its glycosyltransferase, TibC, is functionally interchangeable with AAH (Moormann et al., 2002). Both AIDA-I and TibA mediate bacterial aggregation and biofilm formation through self-association, but this function is independent of glycosylation (Sherlock et al., 2004, 2005).

Some AT proteins exhibit functional redundancy, such that deletion of individual AT genes often does not give rise to a measurable phenotype. As such, we chose to investigate the possible function of EhaJ, and the potential role of glycosylation by EgtA, in an *E. coli* MG1655*flu* strain, which is unable to mediate the classical cell aggregation and biofilm phenotypes associated with Ag43 expression (Reisner et al., 2003). Western blotting indicated that both genes were expressed in this background and that the EhaJ protein has a molecular mass of approximately 155 kDa on SDS-PAGE. However, the molecular mass of EhaJ was approximately 110 kDa when expressed in the absence of EgtA, suggesting that glycosylation of EhaJ is responsible for an approximately 45 kDa shift and that the genes encoding these two proteins are functional. Glycosylation of the 155-kDa protein was confirmed using the glycoprotein-specific Glycoprofile III

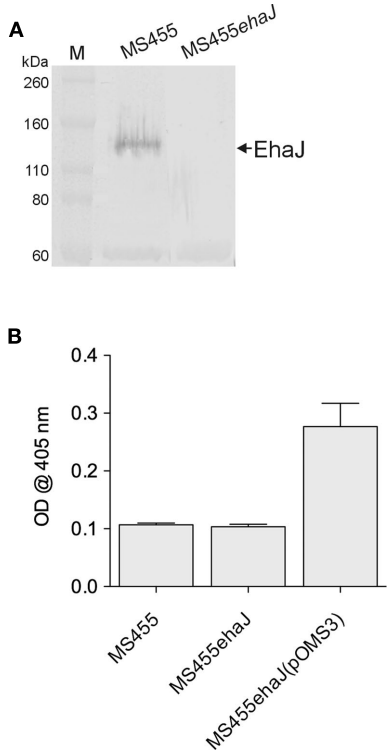

FIGURE 5 | (A) Western blotting with rabbit antiserum specific for the passenger domain of demonstrates expression of EhaJ in wild-type EPEC strain MS455 and loss of EhaJ in MS455*ehaJ*. Molecular mass (kDa) for the protein markers (M) [Novex Sharp Standards, Invitrogen] is shown on the right. **(B)** Wild-type strain MS455 did not bind strongly to the MaxGel protein mixture in an ECM binding assay and inactivation of EhaJ in strain MS455ehaJ did not reduce binding to ECM proteins in this assay ($P = 0.55$). However, over-expression of *ehaJ* (but not *egtA*) in MS455*ehaJ*(pOMS3) lead to a small but reproducible increase in binding to the MaxGel protein mixture ($P = 0.008$). An unpaired *t*-test was used for statistical comparisons using GraphPad Prism 5.

stain. Additionally, immunofluorescence microscopy confirmed that EhaJ was expressed on the bacterial surface.

Several AT proteins mediate cell aggregation and biofilm formation independent of glycosylation, including Ag43, TibA, AidA (Sherlock et al., 2006), and the recently described EhaA (Wells et al., 2008). EhaJ differs from AIDA-I, TibC, and Ag43 in that the passenger domain does not disassociate from the translocation domain following brief heat treatment and induction of EhaJ expression in a K12*flu* background did not result in enhanced bacterial aggregation or biofilm formation in the absence of EgtA-mediated glycosylation. Most interestingly, EhaJ is able to promote biofilm formation when co-expressed with EgtA, demonstrating that this function is dependent upon glycosylation.

Further investigation of the function of EhaJ indicated that it mediates adherence to several ECM proteins. The apparent specificity of EhaJ binding to collagen I, collagen II, collagen III, collagen V, fibronectin, fibrinogen, and laminin is in line with studies on some other AT proteins. For example, EhaB mediates adherence to collagen I and laminin, but not collagen III, collagen IV or fibronectin (Wells et al., 2009), and Tsh (from avian pathogenic *E. coli*) mediates adherence to fibronectin and collagen IV, but not laminin (Kostakioti and Stathopoulos, 2004). Thus, it is clear that while many ATs bind a range of ECM proteins, the specificity of each AT differs, which likely reflects or influences the tissue tropism and pathogenicity of *E. coli* strains. We did not observe any significant difference in the adherence pattern of *E. coli* MS427(pOMS2) and *E. coli* MS427(pOMS3), suggesting that glycosylation of EhaJ does not affect adherence to these ECM proteins. However, it should be noted that small differences in adherence may have been masked by the over-expression of EhaJ, and thus differences may become apparent at lower EhaJ expression levels. Analysis of binding of the wild-type MS455 strain to ECM proteins indicated that this strain does not bind strongly to these proteins when grown in LB broth and deletion of *ehaJ* did not significantly reduce the level of binding. However, the strain was able to bind strongly when *ehaJ* was over-expressed under control of the araBAD promoter, suggesting that a low level of expression of *ehaJ* in the wild-type strain may account for this difference. Studies into the regulation of expression of *ehaJ*, and *egtA*, are therefore necessary to determine the growth conditions under which these genes are expressed to facilitate further functional studies.

Until relatively recently it was thought that only eukaryotic species glycosylate proteins, however many bacterial species are now known to possess N- and O-linked protein glycosylation systems (Nothaft and Szymanski, 2010). The targets of these systems tend to be cell-surface structures such as pili and flagella, in addition to AT proteins. EhaJ represents a new AT protein that mediates adherence to a set of specific extracellular matrix proteins and glycosylation of EhaJ appears to be necessary for its ability to promote biofilm formation. Future studies will explore the regulation of *ehaJ* and *egtA* to determine the growth conditions under which these genes are expressed and thus the potential significance of this glycosylated AT protein to virulence.

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Ribosomal protein S3: a multifunctional target of attaching/effacing bacterial pathogens

Xiaofei Gao and Philip R. Hardwidge*

Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, Kansas City, KS, USA

Edited by:
Elizabeth L. Hartland, University of Melbourne, Australia

Reviewed by:
Elizabeth L. Hartland, University of Melbourne, Australia
Toru Tobe, Osaka University, Japan

****Correspondence:***
Philip R. Hardwidge, Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160, USA.
e-mail: phardwidge@kumc.edu

The extraribosomal functions of ribosomal proteins have drawn significant recent attention. Ribosomal protein S3 (RPS3), a component of the eukaryotic 40S ribosomal subunit, is a multifunctional protein that regulates DNA repair, apoptosis, and the innate immune response to bacterial infection. Here we the review the latest findings about RPS3 extraribosomal functions, with special emphasis on their relation to microbial pathogenesis and enteropathogenic *Escherichia coli*.

Keywords: apoptosis, DNA repair, EPEC, extraribosomal function, NF-κB, NleH1, ribosomal protein S3

INTRODUCTION

Ribosomal proteins function not only in protein translation, but also in multiple extraribosomal activities (Blumenthal and Carmichael, 1979). These functions include, but are not limited to, DNA repair, cell death, inflammation, tumorigenesis, and transcriptional regulation (Warner and McIntosh, 2009). Here we focus on a eukaryotic 40S ribosome component, the ribosomal protein S3 (RPS3), and its emerging regulatory roles in DNA repair, apoptosis, and pro-inflammatory signaling during bacterial infection. We propose that RPS3 may play a central role in regulating numerous aspects of host–pathogen interactions.

RPS3 AND MICROBIAL PATHOGENESIS

Ribosomal protein S3 has been directly and indirectly implicated in host–pathogen interactions. A clone of human RPS3 was obtained in a yeast three-hybrid screen designed to identify proteins that bind the 3′ untranslated region (UTR) of hepatitis C virus (Wood et al., 2001). Suppression subtractive hybridization studies of mast cell gene expression modulated by *Pseudomonas aeruginosa* suggested that RPS3 might be involved in *P. aeruginosa* pathogenesis (Sun et al., 2005). RPS3 expression levels may also be important to mouse resistance to the H5N1 influenza virus (Boon et al., 2009).

The NF-κB family of transcription factors regulates the expression of genes involved in a variety of cellular functions such as immune responses and cellular proliferation (Lenardo and Baltimore, 1989). NF-κB is normally sequestered in the cytoplasm by inhibitory IκB proteins that mask NF-κB nuclear localization signals (Hacker and Karin, 2006). After a cell recognizes a pathogen-associated molecular pattern (PAMP), the IκB kinase (IKK) complex is activated and subsequently phosphorylates the IκBs, leading to their ubiquitination and degradation by the 26S proteasome, permitting NF-κB subunits to translocate into the nucleus to function in transcription.

It was recently discovered that RPS3 is also inducibly associated with and phosphorylated by IKKβ on serine 209 (S209) in response to NF-κB pathway activation (Wan et al., 2011). This phosphorylation event is essential to the nuclear translocation of RPS3, after it associates with importin-α (Wan et al., 2011). Affinity purification experiments had also revealed that RPS3 interacts with the p65 NF-κB subunit through its K homology (KH) domain (Wan et al., 2007; **Figure 1**).

After entering the nucleus, NF-κB binds to κB sites within target gene promoters and regulate transcription by recruiting co-activators/repressors (Wan et al., 2007). This newly discovered NF-κB subunit, RPS3, guides NF-κB to specific κB sites by increasing the affinity of the p65 NF-κB subunit for a subset of target gene promoters (Wan et al., 2007). Likewise, silencing RPS3 expression alters a subset of NF-κB signal transduction pathways. RPS3 thus provides for selective NF-κB recruitment to specific promoters and tailors cellular transcriptional responses to specific stimuli. Interestingly, RPS3 also forms a complex with NF-κB in human islet cells after stimulation with IL-1β (Mokhtari et al., 2009).

The function of type III secretion system (T3SS) effector proteins has been a subject of intense research in recent years (Dean and Kenny, 2009). Some effectors (e.g., NleB, NleC, NleD, NleE, NleH) are key modulators of the innate immune system of intestinal epithelial cells, especially pathways regulated by NF-κB. For example, NleC is a protease that cleaves the NF-κB p65 subunit (Marches et al., 2005; Yen et al., 2010; Baruch et al., 2011; Muhlen et al., 2011; Pearson et al., 2011). NleD cleaves the c-Jun N-terminal kinase (JNK) thus blocking activator protein-1 (AP-1) activation (Baruch et al., 2011). NleE inhibits both p65 nuclear translocation and IκBα degradation (Newton et al., 2010) to block NF-κB activation, in conjunction with NleB (Nadler et al., 2010; Newton et al., 2010).

During attaching/effacing (A/E) pathogen infection, the T3SS effectors NleH1 and NleH2 bind to the N-terminus of RPS3 after their translocation into host cells (Gao et al., 2009). NleH1, but not NleH2, inhibits the nuclear translocation of RPS3, consequently inhibiting the transcription of genes encoding pro-inflammatory

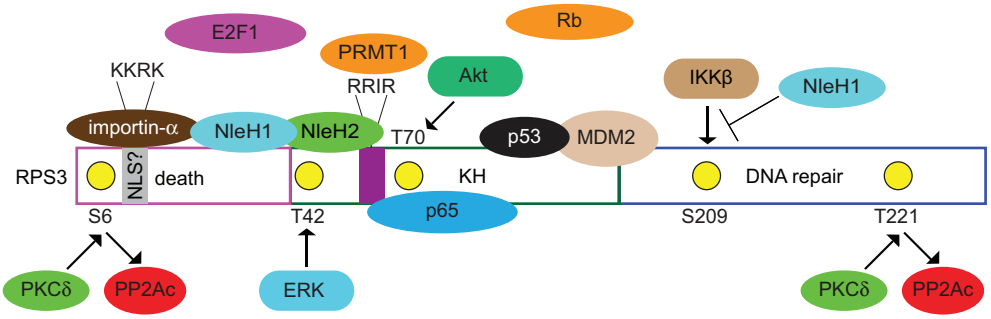

FIGURE 1 | Known and postulated interactions between RPS3 and mammalian or bacterial proteins. Specific RPS3 phosphorylation sites and the protein kinases responsible (PKCδ, ERK, Akt) are indicated. NleH1 and NleH2 are *E. coli* virulence proteins. NleH1 inhibits the IKKβ-mediated phosphorylation of RSP3 S209. Figure is not drawn to scale and specific protein–protein interactions and/or binding interfaces should not be inferred, except where specifically indicated in the text.

cytokines, such as IL-8 and TNF-α, indicating that pathogens target RPS3 to inhibit host immune defenses (Gao et al., 2009).

NleH1 functions by inhibiting the IKKβ-mediated phosphorylation of RPS3 S209 (Wan et al., 2011). NleH1 is an autophosphorylated Ser/Thr protein kinase with an active site at lysine 159 (K159; Gao et al., 2009). While the kinase substrate for NleH1 is not yet known, it does not appear to phosphorylate either IKKβ or RPS3. However, NleH1 kinase activity is required to inhibit IKKβ from phosphorylating RPS3, as mutating the NleH1 K159 residue to alanine (K159A) prevented NleH1 from inhibiting RPS3 S209 phosphorylation, both *in vitro* and in cell culture models (Wan et al., 2011). Studies of gnotobiotic piglets infected with *Escherichia coli* O157:H7 also demonstrated that RPS3 S209 phosphorylation is inhibited by NleH1 *in vivo*, possibly to benefit bacterial colonization and transmission (Wan et al., 2011).

It is interesting that IKKβ activation and IκBα degradation appear to be unaffected by NleH1 (Wan et al., 2011) suggesting that it may be beneficial for the pathogen to attenuate the transcription of RPS3-dependent, but not all NF-κB-dependent target genes. It will be important to consider how the apparently selective alteration of NF-κB activity achieved by NleH1 is coordinated with the other enteropathogenic *E. coli* (EPEC) effectors targeting the NF-κB pathway.

NleH also functions in preventing host cell apoptosis through a mechanism likely to be independent of its interaction with RPS3 (the role of RPS3 in regulating apoptosis will be discussed below). The EPEC effector EspF disrupts host mitochondrial membrane potential and induces the degradation of the anti-apoptotic protein Abcf2 (Nougayrede et al., 2007). Despite the pro-apoptotic function of EspF, EPEC does not induce a large degree of apoptosis, suggesting that other effectors may have anti-apoptotic function. Indeed, an EPEC mutant deleted for both *nleH1* and *nleH2* reduced host cell survival as compared with wild-type EPEC infection (Hemrajani et al., 2010).

Yeast two-hybrid studies subsequently revealed that NleH1 binds to the Bax inhibitor-1 (BI-1) protein (Hemrajani et al., 2010). This result is interesting because the intrinsic pro-apoptotic pathway involves the activation of Bcl-2-homology 3-only (BH3) proteins, as well as the oligomerization of Bak/Bax proteins. Transfecting NleH1 prevented caspase-3 activation (Hemrajani et al., 2010), as well as *Clostridium* TcdB-induced apoptosis (Robinson et al., 2010). Interestingly, a pro-apoptotic Bcl-2 protein, the BH3 interacting domain death agonist (BID) was recently shown to interact with the nucleotide-binding oligomerization domain-containing proteins NOD1 and NOD2, as well as the IKK complex, thus integrating apoptosis and NF-κB signaling (Yeretssian et al., 2011).

NleH1 also interacts with the Na^+/H^+-exchange regulatory factor 2 (NHERF2) at the plasma membrane (Martinez et al., 2010). Because over-expressing NHERF2 reduces the anti-apoptotic function of NleH1, it has been suggested that NHERF2 may serve as a plasma membrane sorting site to bind bacterial effector proteins away from other host targets (Martinez et al., 2010).

DNA REPAIR

It was determined, after the *Drosophila melanogaster* RPS3 cDNA was cloned (Wilson et al., 1993), that RPS3 cleaves DNA at apurinic/apyrimidinic (AP) sites of DNA damage (Wilson et al., 1993). The AP site is a DNA lesion which, without removal, can halt mRNA and DNA synthesis and cause cell death (Loeb and Preston, 1986). *Drosophila* RPS3 possesses an N-glycosylase activity and liberates 8-oxoguanine (8-oxoG) DNA lesions generated during oxidative stress (Yacoub et al., 1996; Deutsch et al., 1997). Transforming RPS3 into *E. coli* rescues the H_2O_2 sensitivity of an *E. coli mutM* strain, as well as the alkylation sensitivity of exo III and endo IV *E. coli* mutants (Yacoub et al., 1996). *Drosophila* RPS3 also accelerates the repair of 8-oxoG lesions in both human and mouse cell extracts (Cappelli et al., 2003).

Both human (Hegde et al., 2004a) and yeast (Jung et al., 2001) RPS3 are also involved in DNA repair. Over-expressing yeast RPS3 overcomes both the osmotic and oxidative stress sensitivity normally observed in a *yar1* mutant, a gene encoding an ankyrin-rich repeat protein that serves a stress response function (Loar et al., 2004). The RPS3 gene is found in a single copy in *Saccharomyces cerevisiae* and its disruption yields non-viable haploid spores (Fingen-Eigen et al., 1996). Human RPS3 binds tightly to both AP and 8-oxoG sites (Hegde et al., 2004b), but appears not to possess its own glycosylase activity (Kim et al., 2005a). Human RPS3 instead functions by binding to and stimulating the activities

of a uracil-DNA glycosylase (Ko et al., 2008), as well as the base excision repair (BER) enzymes hOGG1 and APE/Ref-1 (Hegde et al., 2004a), both of which are multifunctional proteins with AP endonuclease activity.

Ribosomal protein S3 nuclear translocation and its subsequent participation in DNA repair are governed by several post-translational modifications. While RPS3 contains a putative nuclear localization sequence (AAs 7–10; KKRK), no data are yet available to indicate definitively whether this sequence motif is essential to RPS3 nuclear translocation. RPS3 nuclear translocation in response to DNA damage (Yadavilli et al., 2007) is regulated by the extracellular signal-regulated kinase 1 (ERK1), which phosphorylates RPS3 on T42 (Kim et al., 2005b; **Figure 2**). This phosphorylation is critical to regulating RPS3 nuclear translocation, as an RPS3 T42A mutant is significantly reduced in nuclear abundance, even after extensive DNA damage is induced by H_2O_2 treatment. In contrast, an RPS3 T42D mutant is constitutively localized to the nucleus, even in the absence of DNA damage (Yadavilli et al., 2007).

Protein kinase C delta (PKCδ) is a serine–threonine protein kinase that can function in cellular responses relating to DNA damage (Yoshida, 2007). PKCδ phosphorylates the S6 and T221 residues of RPS3 (Kim et al., 2009a). Chemicals that activate PKCδ [e.g., phorbol myristate acetate (PMA) or H_2O_2] increase both the extent of phosphorylation and the repair endonuclease activity of RPS3 (Kim et al., 2009a). After nuclear translocation, the N-terminus of RPS3 can be bound by the catalytic subunit of protein phosphatase 2A (PP2Ac; Kim et al., 2009b) and it appears that prior phosphorylation of RPS3 on S6/T221 is necessary for this interaction (Kim et al., 2009b).

A recent study showed that EPEC enters crypts of the human colon (Maddocks et al., 2009). Studies with human colorectal cell cultures demonstrated that EPEC downregulates the expression of the mismatch repair proteins MLH1 and MSH2, in a mechanism independent of apoptosis (Maddocks et al., 2009). It will be interesting to determine the extent to which EPEC effector protein interactions with RPS3 might account for this phenotype.

APOPTOSIS

Several ribosomal proteins, including RPS3, regulate apoptosis (Naora et al., 1998; Khanna et al., 2003; He and Sun, 2007). Mutational analysis of RPS3 suggests that RPS3 amino acids 15–26,

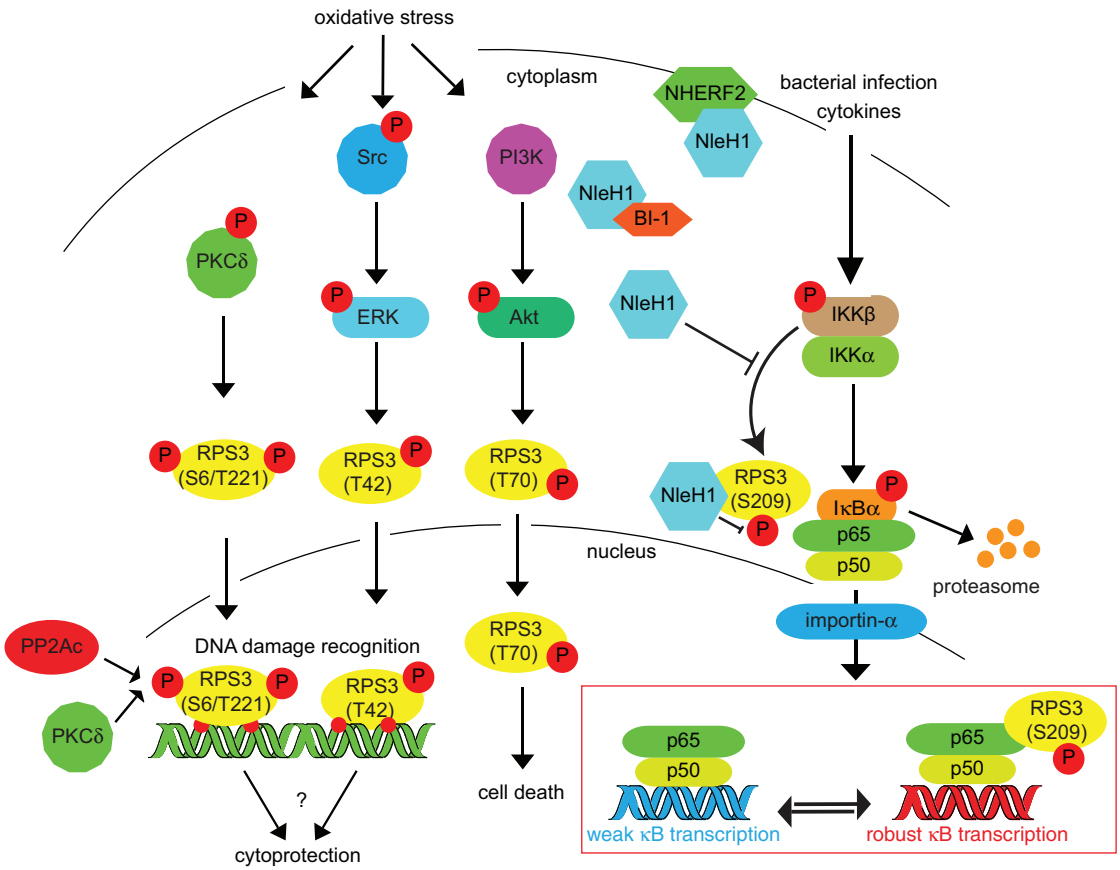

FIGURE 2 | Inducing RPS3 phosphorylation via oxidative stress, DNA damage, and bacterial infection. RPS3 phosphorylation is activated by a variety of cellular stressors. The specific phosphorylation sites and the protein kinases responsible (PKCδ, ERK, Akt) are indicated. Nuclear RPS3 is also bound by the catalytic subunit of protein phosphatase 2A (PP2Ac). IKKβ phosphorylates RPS3 S209 in response to cytokines or bacterial infection. This phosphorylation can be inhibited by the *E. coli* NleH1 protein, which also binds the BI-1 and NHERF2 proteins.

the "death domain," are critical to the function of RPS3 in inducing apoptosis (Jang et al., 2004). Over-expressing an RPS3–GFP fusion induces DNA condensation and promotes the degradation of both the poly (ADP-ribose) polymerase (PARP) and lamin A/C (Jang et al., 2004; Lee et al., 2010), both of which are hallmarks of apoptotic induction. By contrast, depleting endogenous RPS3 rescues cell survival under oxidative stress conditions (Hegde et al., 2007). Caspase-3, -8, and -9 are activated by over-expressing RPS3 in mouse MPC-11 cells, indicating that RPS3-induced apoptosis is likely to be caspase-dependent (Jang et al., 2004).

In addition, either over-expressing or knocking down RPS3 expression levels can lead to apoptosis, suggesting that the total abundance of RPS3 is important to proper cellular function. Mouse embryonic fibroblasts (MEFs) derived from mice engineered to over-express RPS3 display increased levels of DNA damage after oxidative stress, possibly attributable to RPS3 binding to 8-oxoG and blocking BER activities (Hegde et al., 2009). However, in other systems, in the presence of DNA damaging agents (e.g., H_2O_2 and methyl methanesulfonate; MMS), knocking down RPS3 actually leads to increased cell survival, by relieving the RPS3-obstacle to liberating 8-oxoG from damaged DNA (Hegde et al., 2007). Knocking down *C. elegans* RPS3 expression after worms reach adulthood increases lifespan (Curran and Ruvkun, 2007), but the mechanism was not studied in detail.

Ribosomal protein S3 is a substrate for another kinase, Akt (Lee et al., 2010), which can be activated by insulin and pro-survival factors. Activated Akt phosphorylates a variety of cellular proteins involved in the cell cycle, cell survival, and metabolism. Akt phosphorylates the RPS3 T70 residue, also promoting RPS3 nucleus translocation. In this case, T70 phosphorylation is suggested to prevent RPS3-induced apoptosis (Lee et al., 2010). In neuronal cells, RPS3 induces apoptosis by upregulating the expression of pro-apoptotic BH3-only proteins such as Bim and the activator of apoptosis harakiri (Dp5/HRK) by interacting with transcription factor E2F1 (Hershko and Ginsberg, 2004; Lee et al., 2010). Akt-dependent phosphorylation of RPS3 T70 blocks the pro-apoptotic function of RPS3 by inhibiting its interaction with E2F1 while concomitantly enhancing RPS3 endonuclease activity (Lee et al., 2010). These authors also indicate (Lee et al., 2010), in unpublished data, that RPS3 binds to the retinoblastoma (Rb) protein.

A model for RPS3-induced apoptosis has been proposed. Under oxidative stress, RPS3 is phosphorylated by host protein kinases, including ERK, PKCδ, and Akt, consequently translocating into the nucleus to undertake its DNA repair function. Excessive BER may cause irreversible DNA damage and lead to apoptosis (Jang et al., 2004). Thus, increased RPS3 activity at sites of damaged DNA could lead RPS3 to function as an apoptosis signal mediator through a DNA repair enzyme. It is not yet clear what is the mechanism governing cellular apoptosis induced by RPS3 knockdown, though some have speculated that it is related to disrupting protein translation (Lee et al., 2010).

CANCER AND p53

Many studies have identified transcripts and proteins that are differentially expressed in cancer. These proteins include many ribosomal proteins and, among them, RPS3. RPS3 expression is increased in adenocarcinomas and in the majority of adenomatous polyps (Pogue-Geile et al., 1991). Suppression subtractive hybridization also identified RPS3 as over-expressed in a leukemia cell line (Zhu et al., 2003). In contrast, RPS3 appears to be under-expressed in squamous cell lung carcinomas (McDoniels-Silvers et al., 2002).

Ribosomal proteins regulate p53 activity, a tumor suppressor involved in arresting the cell cycle and inducing apoptosis (Sulic et al., 2005; Panic et al., 2006; Chakraborty et al., 2009). Zebrafish with heterozygous mutations in genes encoding 17 different ribosomal proteins are impaired in p53 protein production and develop a rare malignant peripheral nerve sheath tumor (Amsterdam et al., 2004). Disrupting the regulation of p53 protein production may lead to tumorigenesis (MacInnes et al., 2008). p53 Levels are normally regulated by MDM2, which possesses an E3 ubiquitin ligase activity that promotes p53 degradation (Honda et al., 1997). However, during nucleolar stress, ribosomal proteins interact with the acidic zinc finger region of MDM2 (Horn and Vousden, 2008), limiting the function of MDM2.

Ribosomal protein S3 interacts, via its KH RNA-binding domain, with both MDM2 and p53 (Yadavilli et al., 2009). p53 and MDM2 levels normally increase and decrease, respectively, after oxidative stress. By contrast, in cells knocked down for RPS3 expression, p53 levels decrease and the E3 ligase activity of MDM2 is lost (Yadavilli et al., 2009), suggesting RPS3 is important to stabilizing p53.

Ribosomal protein S3 can also interact, via its N-terminus, with a nucleoside diphophate kinase, NM23-H1, that is activated during apoptosis and may function as a tumor suppressor (Kim and Kim, 2006). Over-expressing RPS3 reduces invasion by human fibrosarcoma cells and reduced matrix metalloproteinase 9 (MMP-9) secretion and ERK activation in HT1080 cells (Kim and Kim, 2006). NM23-H1 supresses tumor invasiveness by attenuating Ras-Raf-MEK-ERK signaling (Hartsough et al., 2002). As MMP secretion is regulated by ERK and is thought to be critical to tumor cell invasiveness (Lakka et al., 2002), RPS3 interaction with both NM23-H1 and ERK may play an important role in antagonizing cancer development.

SUMMARY

Ribosomal protein S3 is involved in a broad range of physiological activities. It is reasonable to speculate that interrupting ribosomal protein function via environmental stress or infection will lead to changes in host cell survival. Future studies are likely to reveal additional surprises about the extraribosomal functions of ribosomal proteins and the extent to which these functions are targeted for subversion by the T3SS effectors of A/E pathogens.

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Screening of an *E. coli* O157:H7 bacterial artificial chromosome library by comparative genomic hybridization to identify genomic regions contributing to growth in bovine gastrointestinal mucus and epithelial cell colonization

Jianing Bai*[1,2]*, Sean P. McAteer*[1]*, Edith Paxton*[1]*, Arvind Mahajan*[1]*, David L. Gally*[1] *and Jai J. Tree*[1]*

[1] *Infection and Immunity Division, The Roslin Institute and The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, UK*
[2] *College of Life Science, Hebei Normal University, Shijiazhuang, Hebei Province, China*

Edited by:
John M. Leong, University of Massachusetts Medical School, USA

Reviewed by:
Jennifer Ritchie, Brigham and Women's Hospital, USA
Brian Akerley, University of Massachusetts Medical School, USA
Carolyn J. Hovde, University of Idaho, USA

****Correspondence:***
Jai J. Tree, Infection and Immunity Division, The Roslin Institute and The Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh EH25 9RG, UK.
e-mail: jtree@staffmail.ed.ac.uk

Enterohemorrhagic *E. coli* (EHEC) O157:H7 can cause serious gastrointestinal and systemic disease in humans following direct or indirect exposure to ruminant feces containing the bacterium. The main colonization site of EHEC O157:H7 in cattle is the terminal rectum where the bacteria intimately attach to the epithelium and multiply in the intestinal mucus. This study aimed to identify genomic regions of EHEC O157:H7 that contribute to colonization and multiplication at this site. A bacterial artificial chromosome (BAC) library was generated from a derivative of the sequenced *E. coli* O157:H7 Sakai strain. The library contains 1152 clones averaging 150 kbp. To verify the library, clones containing a complete locus of enterocyte effacement (LEE) were identified by DNA hybridization. In line with a previous report, these did not confer a type III secretion (T3S) capacity to the K-12 host strain. However, conjugation of one of the BAC clones into a strain containing a partial LEE deletion restored T3S. Three hundred eighty-four clones from the library were subjected to two different selective screens; one involved three rounds of adherence assays to bovine primary rectal epithelial cells while the other competed the clones over three rounds of growth in bovine rectal mucus. The input strain DNA was then compared with the selected strains using comparative genomic hybridization (CGH) on an *E. coli* microarray. The adherence assay enriched for pO157 DNA indicating the importance of this plasmid for colonization of rectal epithelial cells. The mucus assay enriched for multiple regions involved in carbohydrate utilization, including hexuronate uptake, indicating that these regions provide a competitive growth advantage in bovine mucus. This BAC-CGH approach provides a positive selection screen that complements negative selection transposon-based screens. As demonstrated, this may be of particular use for identifying genes with redundant functions such as adhesion and carbon metabolism.

Keywords: EHEC, bacterial artificial chromosome, mucus, comparative genomic hybridization, locus of enterocyte effacement, sugar utilization

INTRODUCTION

Enterohemorrhagic *E. coli* (EHEC) strains are associated with serious gastrointestinal disease in humans that can lead to life threatening vascular damage due to the activity of Shiga toxins. The predominant serotypes associated with human infections are O157:H7 and O26:H11 and strains persist in ruminant hosts with humans as an incidental host. It has been demonstrated that EHEC O157:H7 predominately colonizes the terminal rectum of cattle and bacterial multiplication at this site leads to the fecal excretion that is a threat to human health through contamination of bovine food products, produce, and water supplies (Naylor et al., 2003; Chase-Topping et al., 2008). There are a number of EHEC O157:H7 factors that are known to contribute to colonization of cattle at this specific gastrointestinal niche based on both *in vivo* studies and through research on primary epithelial cells cultured from crypts isolated from this rectal site (Chase-Topping et al., 2008). These include the locus of enterocyte effacement (LEE)-encoded type III secretion system (T3SS), various T3-secreted effector proteins, H7 flagellin, and a number of specific adhesins, including F9 fimbriae and autotransporters.

Over the last decade signature-tagged mutagenesis has been applied to extend and confirm genes important for bacterial carriage. This included screening for EHEC O157 and O26 genes important for colonization in cattle (Dziva et al., 2004; Van Diemen et al., 2005). Now with the application of massively parallel sequencing, these studies can be quantified giving exquisite information on the relative significance of each

gene containing a transposon insert that is introduced into the animal (Eckert et al., 2011). This work has highlighted the importance of many of the T3-secreted effector proteins and raised interesting questions about inserts that are an advantage *in vivo*. However, this insertional mutagenesis primarily examines the effects of single genes or individual disrupted operons. Many virulence related phenotypes, such as iron acquisition, carbon utilization, and adherence are encoded redundantly within the genome making them difficult to interrogate with single deletions.

Based on the sequences of multiple EHEC strains that are now available, it is evident that the EHEC pathotype has arisen multiple times by independent acquisition of virulence factors on mobile genetic elements (Ogura et al., 2009). Large horizontally acquired regions, absent from the *E. coli* K-12 genome sequence (designated O-islands, OI), account for a significant proportion of the EHEC genome (Perna et al., 2001; Ohnishi et al., 2002; Zhang et al., 2007) and are likely critical for its niche adaptation encoding factors for nutrient acquisition and adherence. To address what these large regions contribute to the biology of the bacterium, research has been carried out on deletions of OI demonstrating their importance for colonization and persistence (Tree et al., 2011).

The aim of the research presented here was to complement these different screening approaches by generating a bacterial artificial chromosome (BAC) library from an EHEC O157:H7 strain in an *E. coli* K-12 background and then use competition-based assays to select for BAC clones that provide an advantage under *in vitro* conditions relevant to colonization of the bovine host. Comparative genome hybridization on an oligonucleotide microarray was then used to compare the input and output libraries. We have demonstrated that this approach does select for genetic regions with growth and colonization advantages. Several regions of the EHEC genome containing sugar catabolic loci were enriched using this approach and we demonstrate that BAC clones containing hexuronic acid and galactosamine/*N*-acetylgalactosamine catabolism genes increase growth of *E. coli* in bovine terminal rectal mucus. This work raises the possibility of targeting these sugar uptake systems to limit bacterial growth in certain host environments.

RESULTS

GENERATION AND VALIDATION OF AN EHEC O157 BAC LIBRARY

In order to screen large chromosomal fragments that confer virulence phenotypes, a *Hin*dIII BAC library of DNA fragments with an average size of 150 kbp from gDNA isolated from *E. coli* O157:H7 strain Sakai *stx*$^{-}$ (Dahan et al., 2004) was generated in pCLD04541 (Jones et al., 1992) and transformed into *E. coli* DH10B. The BAC library was produced by Lucigen Corporation (Middleton, WI, USA) and was screened for specific sequences by Southern hybridization of colony filters made by the same company.

Type III secretion (T3S) is of central importance for EHEC and is required for colonization of the bovine host. The T3S machine is encoded by the LEE, an approx. 45 kb island inserted into the common *E. coli* backbone. Using a probe designed to hybridize *ecs4562* (encoded within the LEE) we identified 11 BAC clones that contain *ecs4562* and then screened these for clones containing the entire LEE by using PCR primers that amplified across the junctions between the LEE and common backbone (**Table 1**). Clone 3/A2 was found to encode the entire LEE and sequencing of the clone ends from the plasmid indicated that it contained bases 4486393–4639841 of the Sakai chromosome, a 153.4-kbp fragment containing the LEE. To determine if this region was sufficient to confer T3S on *E. coli* DH10B; clone 3/A2 (pBAC3/A2) was grown under T3S permissive conditions and secreted proteins isolated and analyzed for the needle filament protein EspD by western blotting. Secretion of EspD could not be detected (data not shown) in agreement with previous observations (Elliott et al., 1999). To verify that pBAC3/A2 encodes a functional copy of the LEE, a triparental mating using pBAC3/A2 as the donor, pRK2013 (Ditta et al., 1980) as helper and *E. coli* O157:H7 strain TUV93-0 Δ148A Nal^{r} as the recipient was performed. TUV93-0 Δ148A Nal^{r} contains an 8.93-kb deletion within the LEE (from 4677931 to 4686861 as defined for the EDL933 genome) and is deleted for LEE1, LEE2, and most of the LEE3 operon (*escN* onwards are intact) and is unable to export translocon or effector proteins (**Figure 1**; Campellone et al., 2004). Introduction of pBAC3/A2 restored T3S to TUV93-0 Δ148A Nal^{r} (**Figure 1**) indicating that this clone is likely to contain a functional LEE and that the LEE encoded by *E. coli* O157:H7 strain Sakai *stx*$^{-}$ is not sufficient to

Table 1 | Specific strains and plasmids associated with the study.

Strain	Genotype	Source
DH10B	F− *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ−	Lucigen
E. coli O157:H7 strain Sakai	*stx2A*::*kan* Δ*stx1A*′	Dahan et al. (2004)
E. coli O157:H7 strain TUV93-0	ΔBP933-W ΔCP933-V	Campellone et al. (2004)
E. coli O157:H7 strain TUV93-0 Δ148A	TUV93-0 ΔLEE1-3 (4677931–4686861 as defined for the EDL933 genome)	Campellone et al. (2004)
3/A2	DH10B pBAC3/A2	This study
I12	DH10B pV41 containing *agaWEFA* and *uxaC* fragment	This study
Plasmid	**Genotype**	**Source**
pV41	Bacterial artificial chromosome	Lucigen
pRK2013	Mobilization helper plasmid	Ditta et al., 1980
pBAC3/A2	pV41 containing the LEE (4486393–4639841 as defined for the Sakai genome)	This study

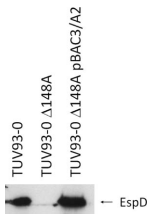

FIGURE 1 | Complementation of T3S in TUV93-0 Δ148A. The LEE containing clone pBAC3/A2 was introduced into the TUV93-0 Δ148A containing a partial deletion of the LEE. A western blot of secreted proteins isolated from TUV93-0 (wt), TUV93-0 Δ148A, and the complemented strain TUV93-0 Δ148A pBAC3/A2 are shown.

confer detectable T3S on a non-pathogenic K-12 isolate such as DH10B. The extra-LEE elements that confer T3S in Sakai are still unclear.

SELECTION OF CLONES THAT ADHERE TO BOVINE PRIMARY CELLS

E. coli O157:H7 colonize the terminal rectal tissue of cattle and replication at this site and associated mucus leads to excretion in the feces and contamination of the environment and the food-chain. High levels of colonization at the terminal rectum are correlated with high numbers of organisms shed into the environment and a higher probability of causing human disease (so called "supershedders"; Matthews et al., 2006). Colonization factors that confer adherence to terminal rectal tissues are important potential targets to prevent colonization by vaccination or more direct interventions. In an effort to identify genomic regions of *E. coli* O157:H7 that may confer adhesion 384 BAC clones were used in repeated rounds of adhesion and culture on bovine primary terminal rectal cells. BAC clones were incubated with primary tissue culture for 4 h at 37°C after which the monolayer was washed to remove unattached bacteria and disrupted to recover adherent bacteria. This pool of adherent bacteria was further selected by another two rounds of adherence. Selected bacteria were inoculated into DMEM for a final round of growth to amplify the amount of gDNA for labeling and hybridization.

In order to determine which BAC clones increase adherence to primary tissue culture total gDNA was isolated from the input and output pools and comparative genomic hybridization (CGH) performed. CGH allows screening of the entire output pool for regions of the genome that have been enriched by selection. This data is normalized using a LOWESS regression and can be visualized as a ratio of signal within the input and output pools for each gene (**Figure 2** and **Data files 1,3** in Supplementary Material). To reduce variation between individual genes and to identify large regions (containing potentially overlapping BAC clones) that are uniformly increased a sliding window of 50 genes was used to average the signal for each gene. In line with our earlier observations that the LEE did not confer T3S on DH10B, LEE containing BAC clones were not selected on primary cells and in fact were negatively selected under these conditions. A number of genomic regions were enriched above two-fold in the primary tissue culture selected pools and these regions encode adhesins or other factors known to confer attachment to cultured epithelial cells. These include pO157 (Grys et al., 2005b; Dziva et al., 2007; Ho et al., 2008), the autotransporter *ehaA* (Wells et al., 2008), and type 1 fimbriae (Galfi et al., 1998). Encouragingly, autotransporters that have been demonstrated not to confer adhesion to primary bovine terminal rectal cells, such as *ehaB*, *ehaC*, and *ehaD* were not enriched in this genome-wide screen indicating a level of specificity (Wells et al., 2008). By screening the BAC library by CGH we have also identified two novel regions that are enriched by selection on bovine primary cells, a region encompassing O-island 71 (OI-71), and a region containing OI-90 and 91. We were not able to identify a known adhesin on, or surrounding OI-71 but note that the fimbrial cluster designated *loc9* (indicated by *yehD* in **Figure 2**) is encoded adjacent to OI-90. Previous work on *loc9* has indicated that this gene cluster can be expressed under *in vitro* conditions (Low et al., 2006b) and does provide a colonization advantage in cattle (Low et al., 2006a). These enriched regions provide promising targets for further study.

SELECTION OF CLONES THAT PROVIDE A GROWTH ADVANTAGE IN BOVINE RECTAL MUCUS

The restricted colonization site of EHEC O157 in cattle means that majority of bacterial *in vivo* replication is limited to the rectal epithelium or mucus layer before excretion in feces. EHEC O157 should have evolved to grow efficiently in rectal mucus which is likely to require genetic regions allowing use of specific carbon sources at this site, the capacity to deal with host innate defense products and potentially the production of factors that can restrict the growth of competitive microbes. Our recent work modeling bacterial replication rates required at the terminal rectum to account for the excretion levels measured indicates doubling times between 20 and 30 min (unpublished data), toward the maximum rates established for *E. coli in vitro*. Doubling times measured in bovine rectal mucus diluted in Hanks buffer were between 30 and 40 min (data not shown) supporting the nutritional quality of even the diluted substrate.

To identify genomic regions that provide a selective advantage at the terminal rectum we cultured 384 BAC clones in bovine terminal rectal mucus and screened output pools by CGH. Four regions were strongly enriched by culturing in mucus and contain OIs 20, 115, 126, and 175 (indicated in (**Figure 3** and **Data files 2,4** in Supplementary Material). *lacZ* is encoded adjacent to OI-20 and is inactivated in the *E. coli* background DH10B. Lactose is reported to be absent from mucus, but the metabolic pathway for metabolism of lactose is induced by culturing on murine mucus (Chang et al., 2004; Fabich et al., 2008). The region adjacent to *lac* also contains the *mhp* gene cluster allowing bacterial breakdown of 3-(3-hydroxyphenyl)propionic acid. Phenylpropanoic and phenylpropenoic acids and their derivatives are common in the environment, arising as breakdown products of lignin and other plant-derived flavonoids and phenylpropanoids (Torres et al., 2003). Adjacent to this region and central to the selected fragments, is also a two component regulatory system for sensing hexose phosphate and an associated hexose phosphate transporter (*z0461–z0463*).

The region containing OI-175 was also enriched and contains a lesion in the restriction–modification system *hsdR* in DH10B. It seems unlikely that *hsdR* would confer a growth advantage

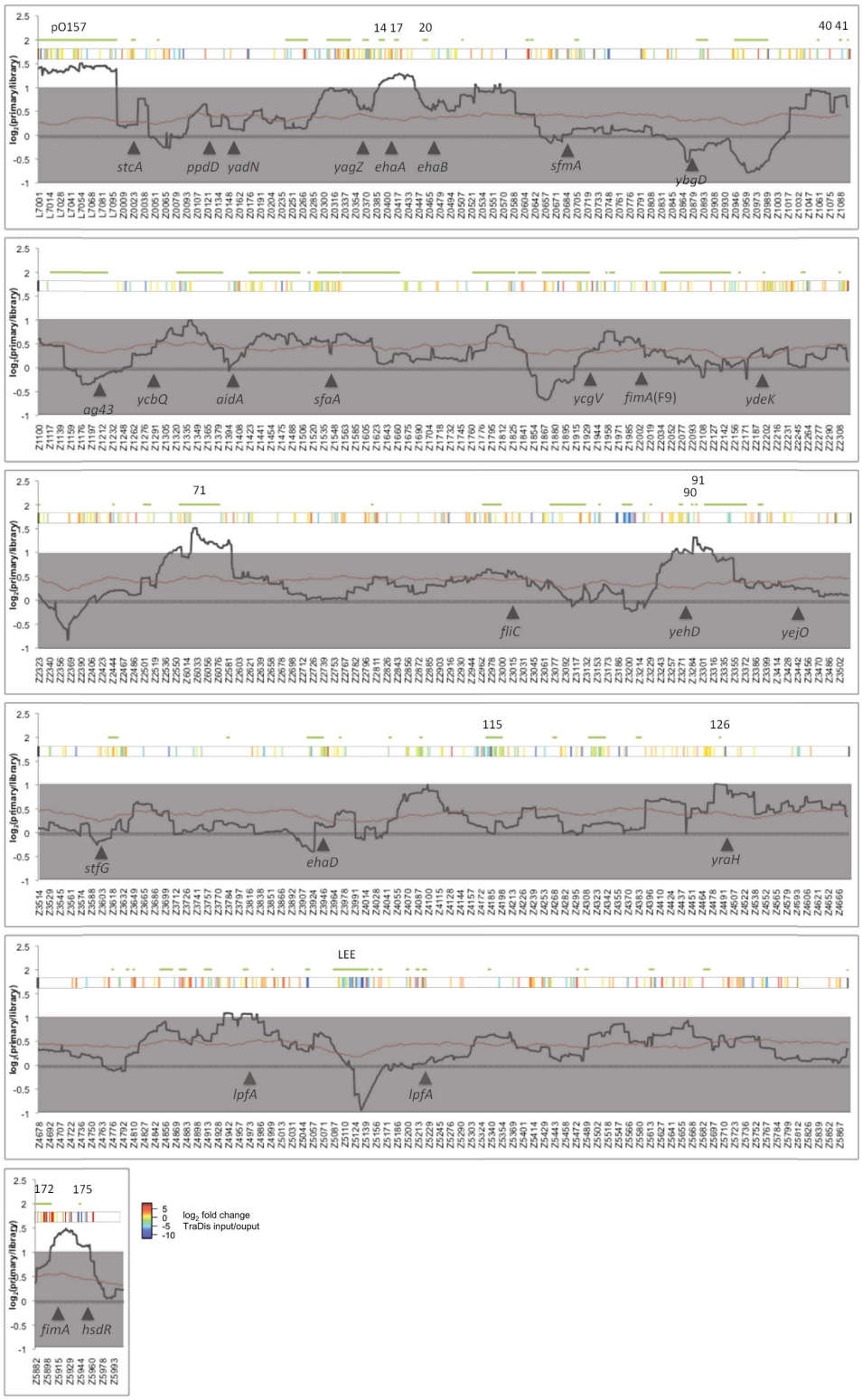

FIGURE 2 | Bacterial artificial chromosome-CGH of primary tissue culture. *E. coli* O157:H7 strain Sakai BAC clones with increased adherence were selected by multiple rounds of adherence to primary bovine terminal rectum cells. Total gDNA from output (adherent) and input (384 clones from the library) pools were subjected to CGH analysis. The ratio of output and input were normalized and a sliding average (window = 50 genes) applied. The black solid line indicates average ratio (output/input), the red line indicates the average *p* value and gray shading indicates an arbitrary two-fold cut-off for significance. Green lines and numbers above the plot indicate the location of O-islands. Solid arrowheads and designations indicate select genes implicated in adherence of *E. coli*.

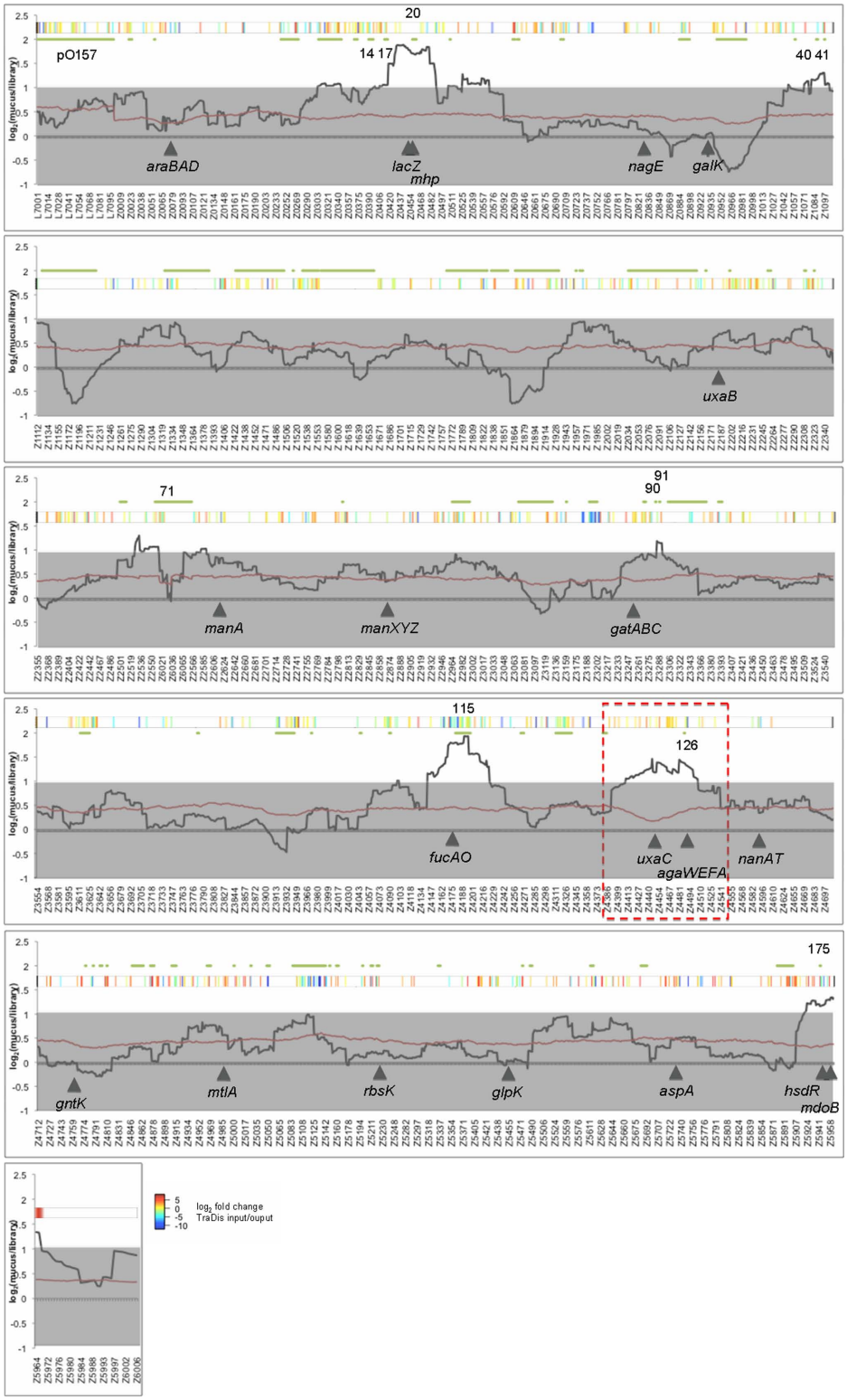

FIGURE 3 | Bacterial artificial chromosome-CGH of mucus-cultured clones. *E. coli* O157:H7 strain Sakai BAC clones were selected cultured were selected by multiple rounds of growth in bovine terminal rectal mucus. Total gDNA from output (mucus grown) and input (384 clones from the library) pools were subjected to CGH analysis. The ratio of output and input were normalized and a sliding average (window = 50 genes) applied. The black solid line indicates average average ratio (output/input), the red line indicates the average *p* value and gray shading indicates an arbitrary two-fold cut-off for significance. Green lines and numbers above the plot indicate the location of O-islands. Solid arrowheads and designations indicate select genes implicated in sugar utilization of *E. coli*. The red dashed box indicates the region selected for further confirmation (see **Figure 4**).

in mucus and this region may contain novel genes involved in mucus growth. For example at the start of the selected region is *gntP*, a fructuronate transporter and central to the region is phosphoglycerol transferase I (*mdoB*).

Two regions contained genes that are known to play an important role in EHEC catabolism of mucus sugars and colonization of cattle. *fucAO* are encoded adjacent to OI-115 and confer a selective advantage on wild-type *E. coli* in mucus when cultured in the presence of an isogenic *fucAO* mutant (Fabich et al., 2008). A strong colonization defect has also been observed in a *fucAO* mutant during cattle colonization (Snider et al., 2009). Similarly, the uronate isomerase *uxaC* (required for hexuronate catabolism) is encoded adjacent to OI-126 and confers a growth advantage on wild-type cells (Fabich et al., 2008). This region also encodes the *agaWEFA* cluster that is required for catabolism of galactosamine and *N*-acetylgalactosamine (GalNAc) and is inactivated in commensal *E. coli* K-12 strains. Both sugars are present in mucus and support growth of EHEC. The presence of the *agaWEFA* cluster has been reported not to confer a significant advantage on wild-type cells *in vitro* however an *agaWEFA* mutant is outcompeted during cattle colonization with significantly less CFU found in terminal rectal mucus (Snider et al., 2009). Enrichment of regions containing *fucAO* and *uxaC/agaWEFA* supports the validity of our results and the ability to BAC-CGH to identify phenotypically relevant regions of the chromosome.

To further confirm our CGH data we have isolated BAC clones that contain *ecs4487* (containing Sakai specific sequence within the *uxaC/agaWEFA* region) and determined their growth phenotype relative to the BAC vector in mucus. A clone containing the *uxaC/agaWEFA* region was identified, designated I12. Both I12 and the BAC vector only (DH10B pV41) strain were marked with Nal^r to allow selection. In reciprocal experiments, the Nal^r tagged I12 or pV41 strain was inoculated at equal CFU into bovine mucus with Nal^s pV41 or I12 respectively. After 24 h culturing in mucus the total CFU/milliliter increased from an average 4.4×10^5–6.5×10^7 (**Figure 4**). During these seven generations the I12 clone was able to outcompete the control vector, increasing from 42 and 43 to 74 and 72% of the total population in reciprocal experiments ($p < 0.05$ in both experiments using a Student's *t*-test). These results confirm that the *uxaC/agaWEFA* region identified in our BAC-CGH screen can confer a selective advantage during growth in bovine mucus.

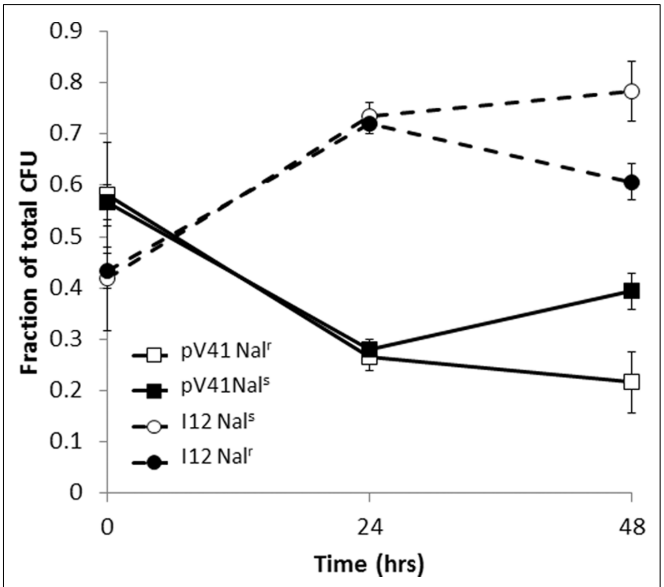

FIGURE 4 | Growth of clone I12 in bovine terminal rectal mucus. Clone I12 encodes the positively selected region containing *uxaC* and *agaWEFA* (see **Figure 3**). DH10B control (pV41) and I12 were tagged with Nal^r and competed against reciprocal Nal^s strains in bovine rectal mucus. CFU/ml of total and Nal^r cells were recorded over 48 h.

DISCUSSION

Based on the sequences of multiple EHEC strains that are now available, it is evident that the EHEC pathotype has arisen multiple times by independent acquisition of virulence factors on mobile genetic elements (Ogura et al., 2009). These horizontally acquired regions add over 1 Mb of extra genetic information to the "core" genome present in non-pathogenic *E. coli* K-12 and encode colonization factors including the T3SS, associated secreted effector proteins, fimbriae, and Shiga toxins (Hayashi et al., 2001; Perna et al., 2001; Ogura et al., 2009). Therefore these often large, horizontally acquired regions account for a significant proportion of the EHEC genome (Ohnishi et al., 2002; Zhang et al., 2007). In an effort to define elements within the EHEC genome that confer a selective advantage when colonizing bovine terminal rectal tissue we have generated a BAC library from a derivative of *E. coli* O157:H7 strain Sakai. This library contains a total of 1152 clones with an average fragment size of 150 kbp and provides 10–15 times coverage of the Sakai genome. This library is likely to be of use for complementing large deletions such as those generated by deletion of entire OIs and we have demonstrated that clones from this library can be selected under *in vitro* colonization relevant conditions identifying regions encoding both established and novel colonization factors. By using CGH to screen output pools we were able to assay enrichment of sequences throughout the genome. This technique may also yield higher resolution of sequences encoding relevant phenotypes than the more traditional approach of isolating individual clones, as overlapping BAC clones will increase the signal recovered for the relevant region.

Using primary bovine rectal tissue culture to select for BAC clones that confer adhesion we have been able to identify regions known to aid attachment to cultured epithelial cells including pO157, *ehaA*, and type 1 fimbriae. The later presents in interesting situation where DH10B may complement the deficiency that has been reported in the O157 type 1 fimbriae phase switch and *fimH* adhesin (Roe et al., 2001; Low et al., 2006a). Additional novel regions were identified containing OI-71 and the fimbrial cluster *loc9*. OI-71 is known to encode T3-secreted effectors (*nleG2-1′*, *nleA*, *nleH1-2*, *nleF*, and *espO1-2*) although it is unclear whether these are responsible for enrichment of OI-71. While a LEE encoding BAC clone could not confer a T3S phenotype on *E. coli* DH10B, it is possible that these proteins could still exert a phenotype, for example by secretion through the flagellar secretion system (Lee and Galan, 2004). Further investigation is required to establish the minimal sequences that are positively selected within the OI-71 and *loc9* encoding regions.

It is evident that positive selection of the BAC library is dependant on appropriate expression of virulence factors in the host strain. This is highlighted by negative selection of the LEE (**Figure 2**), which is required for colonization of bovine terminal rectal tissues but does not confer T3S on DH10B and is strongly negatively selected in our primary tissue culture screen.

The plasmid, pO157 was also selected on primary tissue culture. While not essential for adherence, pO157 confers increased adhesion on O157:H7 (Sheng et al., 2006; Lim et al., 2007) and has been demonstrated to encode at least four loci that are known to increase adherence to epithelial cells. The autotransporter EspP is encoded on pO157 and has been shown to increase adherence to primary bovine terminal rectal cells (Dziva et al., 2007). StcE encodes a protease that cleaves mucin 7 (MUC7), glycoprotein 340 (gp340), and C1-esterase inhibitor (Grys et al., 2005a). MUC7 and gp340 are constituents of the protective glycocalyx covering the epithelium and digestion may allow access to the underlying epithelial cell. StcE is secreted by the type 2 secretion system encoded by the *etp* locus on pO157 and, with YodA, are the only identified substrates. Deletion of *etpC* reduces adherence to HeLa cells in a *stcE* independent fashion indicating that this secretion system is required for function of an as yet unidentified protein(s) involved in adhesion (Ho et al., 2008). ToxB has also been shown to increase adherence, potentially by increasing T3S post-transcriptionally (Tatsuno et al., 2001; Stevens et al., 2004).

Using growth in bovine mucus as a selection for our BAC library we have also selected for multiple regions that contain genes important for uptake and utilization of different sugars present in mucus. Mucus is predominately composed of the glycoprotein, mucin, but also contains smaller fractions of other glycoproteins, proteins, sugars, glycolipids, and lipids, potentially released from epithelial cells sloughed from the intestinal lining (Conway et al., 2007). Mucin is extensively decorated with oligosaccharides that are degraded to monosaccharides by the anaerobic microflora (Conway et al., 2004). A growing body of work has demonstrated that commensal and pathogenic *E. coli* are able to metabolize free gluconate and monosaccharides released from mucin and these likely form a rate limiting substrate for growth (Fabich et al., 2008). Commensal *E. coli* metabolizes at least seven sugars present in mucus: gluconate, *N*-acetylglucosamine, *N*-acetylneuraminic acid (sialic acid), glucuronate, mannose, fucose, and ribose (Chang et al., 2004). *E. coli* O157 can additionally use galactosamine and *N*-acetylgalactosamine (Fabich et al., 2008). Of these, the hexuronates, D-glucuronate and D-galacturonate, are thought to comprise 0.6% of mouse cecal mucus by weight (Conway et al., 2007).

Using CGH to analyze the BAC library after mucus growth, two regions encoding catabolic genes (*fucAO*, *uxaC*, and *agaWEFA*) were selected. These have recently been shown to be important for EHEC colonization of cattle and growth within terminal rectal mucus (Fabich et al., 2008; Snider et al., 2009). Enrichment of these regions lends weight to our CGH analysis of the BAC library pools. We have been able to confirm that screening of our BAC library does produce reliable hits in regions that confer positive selection in mucus by isolating a BAC clone containing the entire *uxaC/agaWEFA* region, designated I12. I12 was able to outcompete DH10B with pCLD04541 when inoculated at equal CFU into mucus after approximately seven generations indicating that the *uxaC/agaWEFA* containing region did confer an enhanced growth phenotype on commensal *E. coli* in mucus.

Genome-wide studies of insertional inactivation events that confer negative selection during host colonization (signature-tagged mutagenesis, TraSH, and TraDIS) have provided a wealth of data about genes that are essential for colonization of host tissue. With the advent of massively parallel sequencing, the depth of coverage and resolution of genes essential for colonization has increased by an order of magnitude (Eckert et al., 2011). Using CGH to analyze an EHEC BAC library selected *in vitro* has provided reliable hits within regions that are positively selected under *in vivo* relevant conditions. This technique is likely to complement existing insertional inactivation screens by provide positive selection of large DNA fragments. This will be of particular use for phenotypes where there is significant redundancy engineered into the genome of pathogens such as adhesion and carbon utilization.

MATERIALS AND METHODS

BACTERIAL STRAINS, PLASMIDS, OLIGONUCLEOTIDES AND MEDIA

The bacterial strains and plasmids used in the study are described in **Table 1**. LB broth was also used (Oxoid). Antibiotics were included when required at the following concentrations: chloramphenicol (12.5 μg/ml), kanamycin (25 μg/ml), tetracycline (12.5 μg/ml), and ampicillin (50 μg/ml).

PREPARATION OF SECRETED PROTEINS AND BACTERIAL FRACTIONS FOR PROTEIN ANALYSES

Bacteria were cultured in 30 ml of MEM–HEPES at 37°C (200 rpm) to an OD_{600} of 0.8 unless specifically stated. The bacterial cells were pelleted by centrifugation at 4000 × *g* for 15 min, and supernatants were passed through filters (0.22 μm). Supernatant proteins were precipitated overnight at 4°C with 10% TCA, and separated by centrifugation at 3220 × *g* for 30 min at 4°C; the proteins were suspended in 100 μl of 1.5 M Tris (pH 8.8). The bacterial pellet was initially suspended in 50 μl sample buffer (Sigma#S3401) and 50 μl molecular biology grade water. Proteins were separated by SDS-PAGE using standard methods and western blotting performed as described previously (Roe et al., 2003).

SELECTION PROCESS WITH BOVINE PRIMARY CELLS AND BOVINE RECTAL MUCUS

Bacterial artificial chromosome library mixture (384 colonies) and pV41 vector only were separately cultured overnight in triplicate in LB. One volume of the above overnight cultures was incubated in 50 volumes of DMEM until the OD_{600} reached 0.6, then adjusted to 0.4. Cultures were diluted 1/100 and then 100 μl/well was transferred into 6-well plates of primary cells. Tissue samples for primary cell culture and isolation of mucus were sourced from a local abattoir. Primary tissue culture was prepared as described previously (Mahajan et al., 2005). Parallel experiments using 1 ml of mucus (verified *E. coli* negative by plating on sorbitol-MacConkey agar) from the first scrap of cattle rectum tissues were also inoculated with 100 μl of bacterial culture. Tetracycline was added to mucus samples to select

for the BAC library or vector, and samples were serially diluted and plated to determine CFU/ml on sorbitol-MacConkey agar at indicated times. For each experiment bacteria with primary cells were incubated for 4 h. Culture medium for primary cells was replaced with DMEM 1 h prior to the above treatment. After unattached bacteria were removed by washing twice with pre-warmed bacterial medium, bacteria from primary cell cultures were collected by mechanical disruption of the monolayer. Bacteria were inoculated and collected from primary tissue culture two further times. Bacterial cells were collected at the end of three rounds of adhesion and subjected to genomic DNA extraction for CGH.

COMPARATIVE GENOMIC HYBRIDIZATION

Genomic DNA was extracted using an Invitrogen ChargeSwitch gDNA Mini Bacteria Kit (Invitrogen). Labeling was carried out using a Bioprime Plus Array CGH Genomic Labeling System (Invitrogen). Protocol for pre-hybridization, hybridization and washing was essentially as previously described for hybridization of cDNA (Roe et al., 2007). Briefly, array slides were washed twice in Wash Buffer II (0.1 × SSC and 0.1% × SDS), each time for 30 s. Slides were transferred in pre-warmed pre-hybridization buffer (0.1% BSA, 0.1% SDS and 5× SSC) for 120 min at 42°C. Slides were washed once in Wash Buffer II and twice in Wash buffer III (0.1 × SSC), and the denatured (95°C for 3 min) hybridization probe mixture (20 pmol of each labeled probe, 0.4 × Ultrahyb, 0.8 × SSC, 90 ng/μl polyA, 10 μg/μl BSA) was added to slides and hybridized overnight at 42°C. Following hybridization slides were washed in Wash Buffer I (0.1% SDS, 2× SSC), Wash buffer II and twice in Wash buffer III. Slides were dried by centrifugation and scanned using an Axon Genepix 4000A scanner (Axon Instruments, Union City, CA, USA). Slide Images were processed using Genepix software and data analyzed using Genespring GX 7.3 (Agilent). Data were normalized using a LOWESS regression. To visualize genome-wide data; a window of 25 genes on either side of each gene was used to average the signal in that region. Data plotted for each gene in **Figures 2** and **3** represents this average. Regions with an average intensity above two-fold across >50 consecutive genes (on average ∼35 kb or ∼25% of the average BAC insert length) were considered significant.

COMPETITION OF DEFINED BAC CLONES IN MUCUS

For competition experiments of individual BAC clones in mucus, spontaneous nalidixic acid resistant (Nal^r) mutants were recovered for both DH10B pCLD04541 and I12 by plating onto LB Nal plates. Competition experiments were performed between DH10B pCLD04541 Nal^s and I12 Nal^r or DH10B pCLD04541 Nal^r and I12 Nal^s. Equal CFU were inoculated into bovine terminal rectal mucus and incubated at 37°C for 24 h. The start and end CFU/ml of each culture was determined by plating on both LB Tet (to select for both strains carrying the BAC vector) or LB Tet/Nal to select for the tagged strain. The relative proportion each strain was determined by subtracting the number of Nal^r colonies from the total Tet resistant population.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/cellular_and_infection_microbiology/10.3389/fmicb.2011.00168/abstract

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Calpain mediates epithelial cell microvillar effacement by enterohemorrhagic *Escherichia coli*

YuShuan Lai*[1], *Kathleen Riley*[2], *Andrew Cai*[1,2,3], *John M. Leong*[1,4]* and *Ira M. Herman*[2,3]

[1] Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA, USA
[2] Graduate Program in Cellular and Molecular Physiology, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, MA, USA
[3] Center for Innovations in Wound Healing Research, Tufts University School of Medicine, Boston, MA, USA
[4] Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA

Edited by:
Elizabeth L. Hartland, The University of Melbourne, Australia

Reviewed by:
Carolyn J. Hovde, University of Idaho, USA
Sabrina Mühlen, University of Melbourne, Australia

***Correspondence:**
John M. Leong, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA, 02111, USA.
e-mail: john.leong@tufts.edu;
Ira M. Herman, Department of Molecular Physiology and Pharmacology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA.
e-mail: ira.herman@tufts.edu

A member of the attaching and effacing (AE) family of pathogens, enterohemorrhagic *Escherichia coli* (EHEC) induces dramatic changes to the intestinal cell cytoskeleton, including effacement of microvilli. Effacement by the related pathogen enteropathogenic *E. coli* (EPEC) requires the activity of the Ca^{+2}-dependent host protease, calpain, which participates in a variety of cellular processes, including cell adhesion and motility. We found that EHEC infection results in an increase in epithelial (CaCo-2a) cell calpain activity and that EHEC-induced microvillar effacement was blocked by ectopic expression of calpastatin, an endogenous calpain inhibitor, or by pretreatment of intestinal cells with a cell-penetrating version of calpastatin. In addition, ezrin, a known calpain substrate that links the plasma membrane to axial actin filaments in microvilli, was cleaved in a calpain-dependent manner during EHEC infection and lost from its normal locale within microvilli. Calpain may be a central conduit through which EHEC and other AE pathogens induce enterocyte cytoskeletal remodeling and exert their pathogenic effects.

Keywords: CaCo-2, calpastat, calpastatin, ezrin, attaching and effacing lesion, microvilli

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) causes serious diarrheal illnesses worldwide. A bacterium that can be found in a variety of ruminants, EHEC can be transmitted to humans by ingestion of contaminated foods. Amongst the various EHEC strains, EHEC serotype O157:H7 has caused most of the serious outbreaks (for review, see Croxen and Finlay, 2010 and Kaper et al., 2004). Symptoms of the disease include severe abdominal cramping, watery diarrhea, hemorrhagic colitis, and in rare cases, hemolytic uremic syndrome (HUS), a triad of hemolysis, thrombocytopenia, and renal failure. HUS, the leading cause of renal failure in children in the US, is caused by systemic absorption of the EHEC toxin, Shiga toxin (Stx), which inhibits protein synthesis (for review, see Tarr et al., 2005).

Enterohemorrhagic *E. coli* is a member of the attaching and effacing (AE) pathogen family, which also includes enteropathogenic *E. coli* (EPEC), an important cause of infantile diarrhea in developing countries, and the mouse pathogen *Citrobacter rodentium* (Mundy et al., 2005; Borenshtein et al., 2008; Frankel and Phillips, 2008). During infection of intestinal epithelial cells, these extracellular pathogens induce dramatic changes in the host cell membrane and cytoskeleton, collectively referred to as AE lesions. At sites of bacterial attachment, surface microvilli are effaced, and bacteria intimately adhere to the host cell surface, appearing to partially "sink" into the mammalian cell. Also characteristic of these lesions is the assembly of striking "pedestals" of filamentous (F-) actin beneath bound bacteria (Caron et al., 2006; Campellone, 2010).

Microvilli are highly organized structures that not only allow for a ∼30-fold increase in the apical surface area of intestinal epithelial, but also serve as sites of robust specialized transport, thereby enhancing enterocytes' ability to absorb water and nutrients (Tyska and Mooseker, 2002; Brown and Mcknight, 2010; Lange, 2010). Loss of microvilli would therefore severely impair absorptive capacity and facilitate diarrheal disease. In addition, AE pathogens that are incapable of generating AE lesions display severe colonization defects and reduced disease phenotypes (Donnenberg et al., 1993; Tzipori et al., 1995; Marches et al., 2000; Tacket et al., 2000; Ritchie et al., 2003), and EHEC mutants defective in stimulating actin pedestal formation fail to expand their initial infectious niche (Ritchie et al., 2008; Crepin et al., 2010).

To generate AE lesions, these pathogens inject effectors into host cells via a contact-dependent type III secretion system (T3SS; Kaper et al., 2004; Croxen and Finlay, 2010). An essential effector is the translocated intimin receptor (Tir), which, after insertion into the host cell apical membrane, binds the bacterial surface protein intimin, thus promoting an intimate connection to the host cell (Kenny et al., 1997). The cytosolic domains of Tir then initiate a signaling cascade that ultimately hijacks a host cell signaling cascade to form filamentous actin pedestals beneath the bound bacteria (Caron et al., 2006; Hayward et al., 2006; Campellone, 2010).

Though both EHEC and EPEC translocate highly related Tir molecules that are required for the formation of morphologically indistinguishable pedestals, the two pathogens trigger F-actin assembly by different signaling pathways (Caron et al., 2006; Hayward et al., 2006; Campellone, 2010). Pedestal formation by EHEC requires a host adaptor, insulin receptor tyrosine kinase substrate (IRTKS) or insulin receptor tyrosine kinase substrate p53 (IRSp53; Vingadassalom et al., 2009; Weiss et al., 2009), and an additional type III-secreted bacterial effector, $EspF_U$, also know as Tir-cytoskeleton coupling protein (TccP), which stimulates the actin nucleation factor neural Wiskott–Aldrich syndrome protein (N-WASP; Campellone et al., 2004; Garmendia et al., 2004). In contrast, EPEC pedestals require recruitment of the host adaptor protein Nck, which in turn binds and activates N-WASP (Gruenheid et al., 2001; Campellone et al., 2002).

While pedestal formation by AE pathogens has been well characterized, much less is known about the mechanisms promoting microvillar effacement. The core bundle of F-actin in a single microvillus is stabilized internally by villin and fimbrin and tethered laterally to adjacent plasma membrane by myosin1A:calmodulin cross-bridges. The bundle is anchored at the base to the terminal web via conventional acto-myosin interactions that ultimately associate with the basolateral membrane domain terminating in adherens and tight junctions where adjacent epithelial cells are tethered to one another (Tyska and Mooseker, 2002; Brown and Mcknight, 2010). In addition, ezrin, an ERM family protein required for microvillar development, is found at this apical cytoskeletal–membrane interface of polarized intestinal epithelia, and is thought to bridge the apical plasma membrane to microvillar F-actin core (Bretscher et al., 2002; Fehon et al., 2010). Core microvillar components are also in constant turnover, making microvilli highly dynamic structures (Tyska and Mooseker, 2002; Brown and Mcknight, 2010). Because of this, there are several potential host proteins that could be targeted by EHEC to alter the cytoskeleton and yield AE lesions. Given the complexity and inherent dynamic nature of microvilli, AE pathogen-induced microvilli effacement likely involves participation of key host adaptor proteins, just as pedestal formation does.

An important class of eukaryotic cytoskeletal regulators are the calpains, Ca^{+2}-dependent proteases that cleave a variety of enzymes and regulatory proteins to modulate cellular function. Calpains are ubiquitously expressed in vertebrates and have been implicated in many important cellular processes, such as regulation of signal transduction, cell spreading and motility, membrane repair, cell death, embryogenesis, and tumor suppression (Potter et al., 1998; Croall and Ersfeld, 2007; Sorimachi et al., 2010). Many reported calpain substrates are involved in regulating the actin dynamics, especially during cellular adhesion and migration (Shuster and Herman, 1995; Potter et al., 1998; Franco and Huttenlocher, 2005; Lebart and Benyamin, 2006; Chan et al., 2010; Kotecki et al., 2010). Several microbial pathogens, promote disease through the inappropriate activation of calpain (Fettucciari et al., 2006; Wang et al., 2008; Goldmann et al., 2009; Dean et al., 2010; Perry et al., 2010; Sumitomo et al., 2010; Zhang et al., 2010). EPEC infection results in an increase intracellular Ca^{+2} levels in mammalian cells (Baldwin et al., 1991), and induces calpain activity in a manner dependent on T3SS (Hardwidge et al., 2004; Dean et al., 2010). We previously showed that calpain controls EPEC-induced enterocyte effacement *in vitro* (Potter et al., 2003).

While calpain's roles in effacement and intestinal barrier disruption have been documented for EPEC, its involvement in EHEC infection has not been characterized. Since comparative analysis of pedestal formation by EPEC and EHEC have revealed that these related pathogens generate actin pedestals by fundamentally different means (Caron et al., 2006; Hayward et al., 2006), we sought to determine if calpain plays an important role in EHEC effacement, as it does in EPEC (Potter et al., 2003). We show, here, that infection of polarized CaCo-2a monolayers by EHEC increased calpain activity, and inhibition of calpain blocked effacement and reduced other morphological manifestations of cell damage. Calpain-dependent ezrin disruption was also observed upon EHEC infection. These results indicate that calpain, perhaps by cleaving ezrin, likely plays a central role in EHEC-induced microvillar effacement. An understanding of the calpain-regulated cytoskeletal remodeling that controls host cell adaptive responses to bacterial challenge may offer innovative therapeutic strategies aimed at preventing EPEC and EHEC pathogenesis.

MATERIALS AND METHODS

ANTIBODIES AND PHALLOIDIN

Alexa 488- and 546-conjugated phalloidin, were obtained from Invitrogen. Primary antibodies used were mouse anti-ezrin (Zymed # 357300), rabbit anti-O157 (Gibco), and goat anti-O157 (Fitzgerald Industries #70-XG13). Secondary antibodies are species-specific Alexa 350-, 488-, and 546-labeled anti-IgG (Invitrogen).

CELL CULTURE

CaCo-2a enterocytes, stably transfected with the calpastatin high over-expression plasmid pRC/CMV-3ΔCSN (HOX) or the pRC/CMV empty vector (CON), have been previously described (Potter et al., 2003). Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1% each penicillin–streptomycin–fungizone (PSF) and L-glutamine. Sub-confluent cultures were maintained in T175 flasks. For experiments, cultures were washed twice with HBSS, released with trypsin–EDTA, and seeded at confluence (approximately 1.5×10^5 cells/cm^2) in tissue cultures vessels precoated with collagen (BD #354236). CaCo-2a cells were then allowed to differentiate for 7–14 days, and fed every 2 or 3 days. For electron microscopy, cells were seeded onto standard plastic six-well, tissue culture plates. For immunofluorescence staining, cells were seeded into 24-well glass bottom tissue culture plates (Mattek #P24G-1.5-13F). For calpain activity, cells were seeded into plastic 96-well black walled tissue culture plates with optically clear bottoms (Potter et al., 2003).

BACTERIAL STRAINS

Table 1 lists bacterial strains used in this study. The wild type, Stx-deficient EHEC strain of serotype O157:H7, strain TUV-93, a derivative of the prototypic *E. coli* O157:H7 strain EDL933, has been previously described. The lack of Shigatoxin production by TUV-93-0 permits the prolonged infection of a wide variety

Table 1 | Bacterial strains used in this study.

Strain	Description	Resistance	Reference
TUV-93	Stx-deficient EHEC serotype O157:H7	N/A	Campellone et al. (2002)
JPN15	EPEC Serotype O127	Amp	Jerse et al. (1990)
TUV-93Δ*escN*	T3SS deficient EHEC mutant derived from TUV-93	Cam	This study

of cell lines without the induction of Stx-mediated cytotoxicity. TUV-93Δ*escN* was generated by lambda Red recombineering as previously described (Murphy and Campellone, 2003).

INFECTION

In preparation for infection, individual colonies from freshly streaked plates were grown in 1 mL of LB with appropriate antibiotics for up to 8 h. Ten microliter of this day culture was transferred to DMEM/100 mM HEPES/antibiotics and incubated overnight at 37˚C with 5% CO_2 to induce T3SS expression. For infection, bacteria were resuspended in DMEM/2% FBS/20 mM HEPES/2 mM glutamine to the following MOI's: EHEC at 500:1, EPEC at 100:1. Higher MOI's were necessary for EHEC infections due to less efficient binding of EHEC to cultured cells; this is a commonly observed phenotype that was noted at least two decades ago (Cantey and Moseley, 1991). Strain TUV-93 also does not adhere to *in vitro* cultured cells as well as EHEC harvested from infected piglets (Brady et al., 2011). The latter observation suggests that the poor cell attachment by *in vitro*-grown EHEC, as were analyzed here (and in virtually all published cell binding studies), reflect the relative lack of a putative adhesin (or adhesins) that is expressed at higher levels when EHEC is growing in the mammalian host.

QUANTIFICATION OF CALPAIN ACTIVITY

Calpain activity was assayed by either one of two fluorogenic calpain substrate kits. For assays using AnaSpec's Calpain Activity Kit (#72150), CaCo-2a cell monolayers are seeded in 96-well TC treated plates with clear bottoms and black walls (Ibidi #89626) 10–14 days prior to use. Cells were infected as described above. Infected monolayers were washed twice with PBS. Fifty microliter of assay buffer was added to each well and incubated at room temperature (RT) for 5–10 min. Cells were scraped with a pipette tip and 50 mL of fluorogenic substrate in assay buffer was added to each well. Plates were spun at 1000 rpm on a tabletop centrifuge ($700 \times g$) for 5 min to reduce bubbles and incubated at RT in the dark for up to 60 min. For assays using Calbiochem's InnoZyme™Calpain Activity Assay Kit, (#CBA054), CaCo-2a cell monolayers are seeded in six-well TC treated plates 10–14 days prior to use. Cells were infected as described above. Infected monolayers were washed twice with PBS, and lysed with CytoBuster™ (EMD #71009) on ice for 5 min. Cell lysates were scraped, transferred into 1.5 mL tubes and spun at 12,000 *g* at 4˚C for 15 min. The supernatants were collected and used immediately for measuring calpain activity per kit instructions or stored at −80˚C. Fluorescence was measured using a Molecular Devices SpectraMax Gemini XS at 354 nm excitation and 442 nm emission for the Anaspec kit and 320 nm excitation and 480 nm emission for the Calbiochem kit, and corrected for background fluorescence (i.e., that of wells with substrate only).

CALPASTAT TREATMENT

Calpastat, a cell-penetrating calpastatin peptide (Croce et al., 1999) was synthesized at the Tufts Peptide Core Facility using solid phase Fmoc chemistry. Peptides were purified by high performance liquid chromatography; molecular mass and purity were confirmed by mass spectrometry. A 25 mM peptide stock was prepared in 100 mM HEPES, pH 7.4 and stored at −20˚C. For treatments, cell monolayers were washed once with HBSS and cell culture media containing Calpastat at the specified concentrations was added. Cells were incubated at 37˚C in 5% CO_2 for 1 h.

SCANNING ELECTRON MICROSCOPY

Cell monolayers were washed twice with HBSS, then fixed by immersion in 2.5% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.2) for a minimum of 2 h at RT. The fixed samples were then washed three times in the same buffer. Following the third wash, monolayers were dehydrated through a graded series of ethanol to 100% and then critical point dried in liquid CO_2. The bottoms of the dishes were cut off and using silver conductive paste, the plastic disks with the cells attached on the surface were affixed to aluminum scanning electron microscopy (SEM) stubs and sputter coated with Au/Pd (80/20). The specimens were then examined using an FEI Quanta 200 FEG MK II scanning electron microscope at 10 kV accelerating voltage. Each specimen was systematically observed at 1000×, 2500×, and 5000× at five separate areas spaced throughout the disk. Images were assessed by four individuals, one of whom was blinded and another of whom was not involved in the study. Assessments were unanimous concerning the presence or absence of effacement. Representative images at 10,000× were taken with disks tilted about 30˚.

IMMUNOFLUORESCENCE MICROSCOPY

For indirect immunofluorescence imaging studies, CaCo-2a cells were seeded at confluence and allowed to differentiate in 24-well glass bottom culture plates (Mattek #P24G-1.5-13F). At the indicated time points following infection, cells were washed with warm DMEM, then fixed in 4% formaldehyde/DMEM for 5 min at RT. Cells were permeabilized with 0.1% Triton buffer for 90 s at RT, washed three times with PBS, and then incubated with the specified primary antibodies for 1 h at RT. Labeled secondary antibodies and/or Alexa 488- or 544-conjugated phalloidin (Invitrogen) were applied for 45 min at RT. Experiments were repeated three times. For each experiment, greater than 5 fields were examined per condition; roughly 20–50 cells per field were viewed/scored. Images were acquired on a Zeiss 200 M inverted microscope with a Hamamatsu cooled-CCD digital camera (ER) and MetaMorph 7.0 imaging software (Molecular Devices Corp, PA, USA).

SUBCELLULAR FRACTIONATION AND WESTERN BLOTTING

Infected and uninfected cells were washed with warm TBS to remove media and unattached bacteria. Extraction buffer containing 40 mM HEPES pH 7.2, 50 mM PIPES, 75 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, Protease Inhibitor Cocktail (1:1000,

Sigma), 1 mM sodium orthovanadate, and 0.1% Triton detergent was applied to the cultures, 0.5 ml per well of a six-well plate, and plates were placed on an orbital shaker at 50 rpm for 10 min at RT. The extraction buffer was then removed to tubes held on ice. The remaining cellular residue was collected with 200 μL boiling hot Laemmli sample buffer (Sambrook and Russell, 2001). All extracts were dialyzed against 4 L distilled water at 4°C for 4 h with one water change at 2 h, using SnakeSkin dialysis tubing, 10 K MWCO (Pierce). Dialyzed samples were snap frozen in liquid nitrogen, lyophilized overnight, and brought up in 200 μL each Laemmli sample buffer. All samples were boiled for 3 min and loaded onto 10% acrylamide gels for SDS-PAGE, followed by transfer to nitrocellulose membranes. Membranes were stained for ponceau to confirm equal protein loading, then probed for ezrin using monoclonal mouse anti-ezrin (Zymed #357300).

RESULTS

CALPAIN ACTIVITY IS INCREASED UPON EHEC INFECTION

To investigate whether calpain activity is affected by EHEC, we used two different commercially available kits that utilized calpain-specific fluorogenic substrates. Compared to uninfected cell monolayers, calpain activity increased after 3 h of infection by EHEC. As reported (Dean et al., 2010), EPEC also induced an increase in calpain activity, while the T3SS deficient EHEC mutant Δ*escn* did not. **Figure 1** shows the results of one representative experiment. Pretreatment of polarized cell monolayers with a cell-penetrating calpastatin inhibitor for 1 h prior to EHEC infection reduced EHEC-induced calpain activation to near uninfected levels (Data not shown).

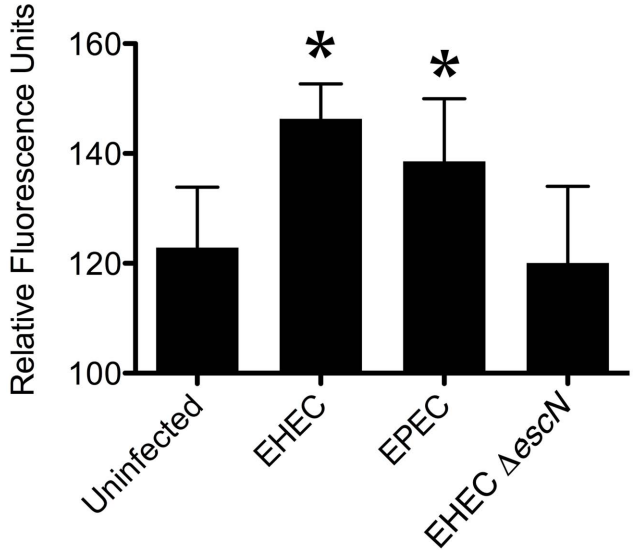

FIGURE 1 | Enterohemorrhagic *Escherichia coli* increased calpain activity during infection. Calpain activity was measured with a fluorogenic, calpain-specific substrates in lysates of uninfected CaCo-2a cells, or of cells infected with EHEC, EPEC and EHEC Δ*escN* for 3 h. Shown are the means (±SE) with (*) indicating significance of $p < 0.05$ by student's *T*-test compared against the uninfected group.

CALPASTAT, A CELL-PENETRATING CALPAIN INHIBITOR PREVENTS EHEC-INDUCED EFFACEMENT

To assess the role of calpain in EHEC-induced effacement, we infected polarized CaCo-2a CON cells with or without pretreatment with Calpastat, a cell-penetrating version of the endogenous calpain inhibitor, calpastatin (Croce et al., 1999; Carragher, 2006). SEM of uninfected control cells revealed distinct microvilli and cellular borders, although some variation in the density and arrangement of microvilli was observed (**Figure 2A**, and data not shown).

When CON cells were infected with EHEC and assessed by SEM at low (1000×) magnification (**Figure 2**) or TEM (data not shown), CON cells suffered severe "blebbing" and rounding, indicating cellular damage (**Figure 2B**). This apparent toxicity was not observed after infection with EPEC (**Figure 2C**), in which cell monolayers looked nearly identical to uninfected monolayers (**Figure 2A**). Pretreatment with 2.5 mM Calpastat for 1 h prior to EHEC infection diminished these effects (**Figure 2D**), suggesting that calpain activity is required for this cellular damage and raising the possibility that calpain may be critical for EHEC-induced cellular pathogenesis.

Higher magnification assessment of monolayers at 5000× to 10,000× revealed that a 2-h infection by EHEC resulted in the predicted microvillar effacement (**Figure 3B**) as compared to uninfected controls (**Figure 3A**). When CON cells were subjected to a 1-h pretreatment with 2.5 mM of Calpastat prior to EHEC infection and assessed by 10,000× magnification SEM, most EHEC-bound cells retained largely intact microvilli (**Figure 3C**), similar

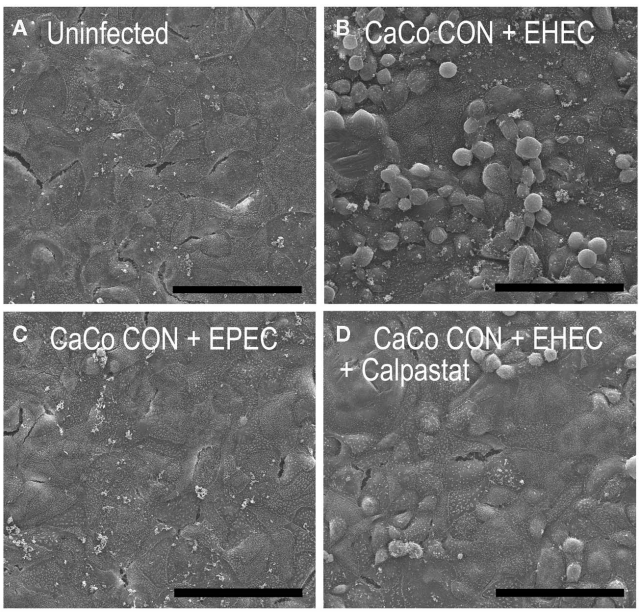

FIGURE 2 | Enterohemorrhagic *Escherichia coli* infection produced additional gross monolayer damage that was diminished by inhibition of calpain. Uninfected CaCo-2a CON monolayers **(A)**, or monolayers infected for 2 h with EPEC **(C)** or EHEC, with **(D)** or without **(B)** a 1-h pretreatment with Calpastat (2.5 μM) were visualized by SEM under 1000× magnification. Scale bars are 100 μm.

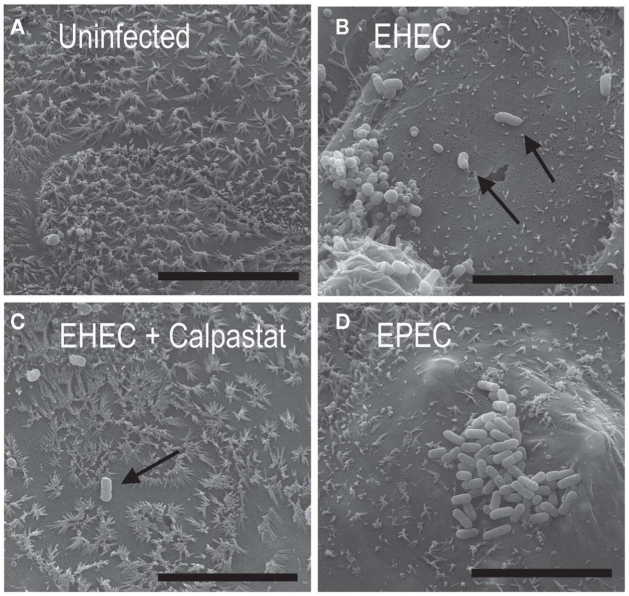

FIGURE 3 | A cell-penetrating calpain inhibitor prevents microvilli effacement by EHEC. Uninfected CaCo-2a CON monolayers **(A)** and monolayers infected for 2 h with EHEC without **(B)** or with **(C)** 1 h pretreatment with 2.5 μM Calpastat, or infected with EPEC **(D)** were visualized by SEM at 10,000× magnification with a 30° tilt. Scale bars are 10 μm.

in quality to uninfected CaCo-2a CON monolayers (**Figure 3A**). As expected, the control infection by EPEC in the absence of Calpastat treatment also revealed effacement (**Figure 3D**).

ECTOPIC EXPRESSION OF CALPASTATIN INHIBITS EFFACEMENT BY EHEC

We previously showed that the calpastatin-overexpressing CaCo-2a cell line, HOX, for which the pRC/CMV vector-transfected CaCo-2a CON line serves as an appropriate "wildtype" control, resists effacement by EPEC (Potter et al., 2003). Like the parent CaCo-2a and vector control CON line, these cells polarize and appear to differentiate, in that they develop trans-epithelial resistance and, by SEM, generate microvilli (albeit morphologically distinct from those on CON cells; Potter et al., 2003). Like CON cells, some cell-to-cell variability with respect to microvillar length and density (data not shown) were observed. HOX microvilli were, intact and in general, shorter than those observed in CON cells, (**Figure 4A**). As reported; these phenotypic differences compared to wild type CaCo-2 cells are likely due to the altered cytoskeletal architecture resulting from disrupted calpain-driven cytoskeletal remodeling (Potter et al., 2003).

When stained for F-actin using fluorescent phalloidin and imaged near the apical surface of the monolayers, the microvillar F-actin core bundles of polarized CON and HOX cells appeared as a punctate fluorescent pattern on the apical surface (**Figures 4C,E**) when viewed "end-on," while the circumferential "belt" of F-actin encircling the apical cytoskeletal–membrane domain could also be readily distinguished. This apical F-actin staining pattern was consistent with the pattern of microvilli observed by SEM

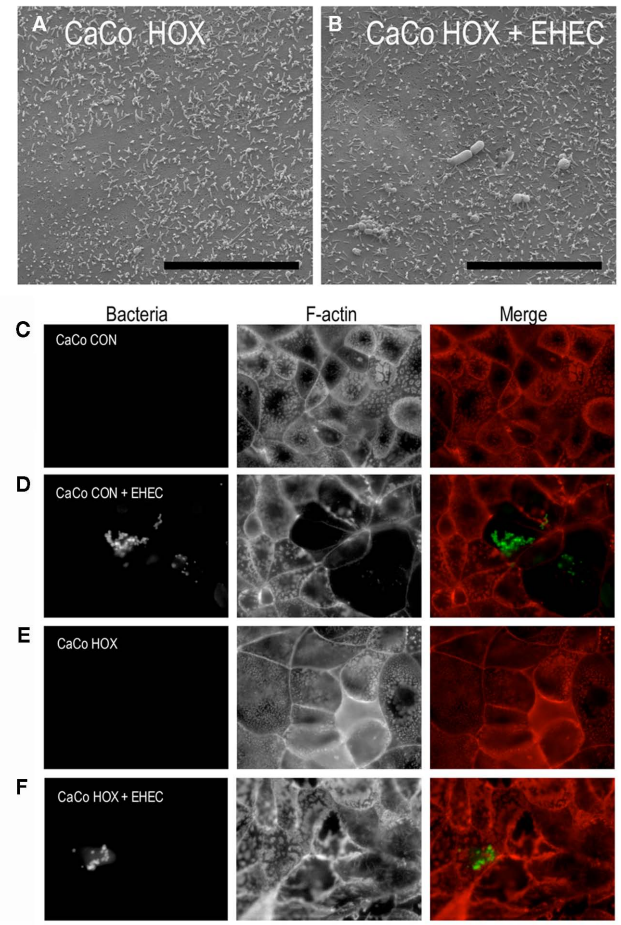

FIGURE 4 | The calpastatin overexpressing cell line, HOX, resisted microvillar effacement by AE pathogens. Uninfected polarized CaCo-2a HOX monolayers **(A)** and polarized HOX monolayers infected for 2 h with EHEC **(B)** were visualized by SEM at 10,000× magnification and with a 30° tilt. Scale bars for SEM's are 10 μm. Polarized control CaCo-2a CON **(C,D)** and calpastatin-overexpressing CaCo-2a HOX cells **(E,F)** were stained for F-actin (red) and EHEC (green) without **(C,E)** or with infection **(D,F)** by EHEC. F-actin was stained using Alexa 568-conjugated phalloidin; EHEC were stained with anti-O157 antibody.

(**Figures 3D** and **4A**). In addition, infection of polarized CON cells by EHEC induced the loss of this punctate fluorescent staining (**Figure 4D**), consistent with the loss of microvilli observed during EHEC-induced effacement as revealed by high magnification SEM (**Figure 3A**). (Note that the EHEC strain used in this study requires 4–6 h of infection to form actin pedestals, and were not detected in the timeframe of the current study.)

In stark contrast to the effacement of CON cells by EHEC, infected HOX cells retained their microvillar-associated punctate F-actin fluorescence staining (**Figure 4F**). The retention of microvillar integrity by HOX cells after 2 h (**Figure 4F**) or 3 h (data not shown) was confirmed by SEM (**Figure 4B** and data not shown). These results indicate that ectopic expression of calpastatin and the concomitant inhibition of calpain renders HOX cells resistant to effacement by EHEC infection.

THE CALPAIN SUBSTRATE EZRIN IS CLEAVED IN RESPONSE TO EHEC INFECTION AND LOST FROM MICROVILLI

We have previously shown that calpain-mediated cleavage of ezrin occurs during cell spreading and migration, a process that, like effacement, involves extensive actin remodeling (Shuster and Herman, 1995; Potter et al., 1998, 2003). Given that EHEC induces calpain activity upon infection of mammalian cells, we tested for ezrin cleavage upon infection by EHEC. Immunoblotting of subcellular fractions of EHEC-infected CaCo-2a CON cells revealed ezrin cleavage (**Figure 5A**). The full-length 80 kDa species was detected in both soluble and insoluble fractions. The cleaved 55 kDa ezrin fragment, which is not associated with the plasma membrane, was detected at very slight levels in the soluble fraction of uninfected CON cells. Endogenous calpain activity is present in all cells, supporting various functions *in situ* such as cellular differentiation, membrane polarization, or cell cycle progression. Therefore a small amount of cleaved ezrin should exist, even in uninfected cells. Infection with EHEC increased this pool of cleaved ezrin. A significant amount of full-length ezrin was still detected in EHEC-infected CON monolayers likely because, as can be seen in **Figures 4D,F**, EHEC does not infect cells uniformly, but rather heavily infects a fraction of cells. Thus, a mixture of infected and uninfected cells contributes to the lysate collected for western blotting. The 55 kDa ezrin cleavage product was not observed in uninfected or EHEC-infected HOX cells, indicating that calpain is required for its generation (**Figure 5A**).

Immunofluorescence staining of ezrin in polarized CaCo-2a CON cells revealed a punctate pattern at the apical surface almost identical to that of microvilli staining by phalloidin (**Figure 5B**). Images taken at the apical surface showed a staining pattern that is consistent with its role in linking the plasma membrane to core microvillar actin bundles (Fehon et al., 2010). To determine if EHEC-mediated cleavage of ezrin correlated with a change in the cellular distribution of ezrin, we stained CON cells for ezrin after EHEC infection. Interestingly, the apical punctate ezrin pattern disappeared throughout the entire infected cell (**Figure 5C**). Cleavage of ezrin would result from its release from the apical surface, thus appearing as a loss of staining in the focal plane imaged. This change in localization was dependent on calpain activity, because calpastatin-overexpressing HOX cells retained their punctate ezrin staining pattern during infection (**Figure 5D**).

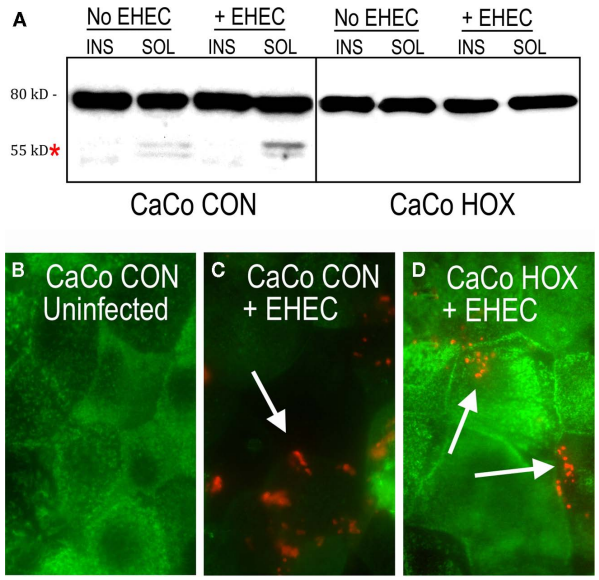

FIGURE 5 | Enterohemorrhagic *Escherichia coli* infection caused calpain-dependent loss of mirovillar ezrin and ezrin cleavage. Lysates from subcellular fractions of polarized control CaCo-2a CON and HOX monolayers with and without 6 h EHEC infection by were probed for ezrin by western blot. Asterisk (or arrow) indicates the ~55 kDa ezrin cleavage product **(A)**. Polarized CaCo-2a CON cells without **(B)** and with 6 h infection by EHEC **(C)** and EHEC-infected polarized HOX monolayers **(D)** were stained for ezrin (green) and EHEC (red). EHEC was detected by anti-O157 antibody.

DISCUSSION

In response to challenge by AE pathogens, intestinal epithelial cells undergo a coordinated and robust remodeling of the apical membrane–cytoskeletal domain (Goosney et al., 2000; Hardwidge et al., 2004; Caron et al., 2006). Highly organized core bundles of actin in the microvilli are disassembled while the apical membrane conforms to newly synthesized actin pedestals. These structural alterations likely disrupt overall epithelial cell function and integrity and contribute to EHEC- and EPEC-induced gastroenteritis and diarrhea. While the process of pedestal formation is well characterized, the mechanisms of microvillar effacement are poorly understood. Previous work indicated that EPEC infection triggers a rise intracellular Ca^{+2} in mammalian cells (Baldwin et al., 1991), and EPEC-mediated effacement requires the Ca^{+2}-regulated host protease, calpain (Potter et al., 2003). In this study, we showed that EHEC infection of CaCo-2a cells induces an increase in calpain activity that is required for EHEC-mediated effacement. By analogy to EPEC, we postulate that type III-secreted effectors play a role in both calpain activation and microvillar effacement (Hardwidge et al., 2004; Dean et al., 2010). In fact, an EHECΔ*escN* mutant, which is defective in type III secretion, did not trigger an increase in calpain activity.

Effacement likely involves disruption of protein–protein and protein–plasma membrane interactions that contribute to microvillar integrity. Calpain is known to target a number of cytoskeletal elements, including the ERM family member ezrin (Shuster and Herman, 1995; Potter et al., 1998; Frame et al., 2002; Franco and Huttenlocher, 2005; Lebart and Benyamin, 2006), which contributes to microvillar integrity by indirectly linking the plasma membrane to the axial actin microfilamentous bundles (Shuster et al., 1996; Bretscher et al., 2002; Saotome et al., 2004). Another gut pathogen, *H. pylori*, triggers calpain-dependent cleavage of ezrin in gastric parietal cells, resulting in redistribution of the protein, distortion of microvillar structure and disruption of apical secretory function (Wang et al., 2008). We showed here that EHEC-mediated effacement of microvilli is accompanied by calpain-dependent cleavage of ezrin and loss of its apical localization.

The targeting of ezrin by AE pathogens is likely to induce widespread changes in the structure and function of intestinal epithelial cells. EPEC triggers the transient formation of filopodia that are significantly destabilized by the expression of a dominant

negative ezrin mutant (Berger et al., 2009). Ezrin has also has been implicated in the assembly of junctional complexes (Pujuguet et al., 2003), and we found that EHEC infection results in a calpain-dependent redistribution away from cell–cell junctions. This relocalization may well have functional consequences on epithelial barrier function, given the observations that the addition of calpain-inhibitory peptides (Dean et al., 2010) and or the expression of a dominant negative ezrin mutant (Simonovic et al., 2001) diminish EPEC-induced disruption of tight junctions and trans-epithelial resistance.

More generally, apical and junctional cytoskeletal domains are functionally integrated: F-actin rich microvillar rootlet structures penetrate the terminal web, making intimate contact with the circumferentially disposed array of filamentous actin, conventional myosins and intermediate filaments, which all join together in the terminal web at adherens and tight, occluding junctions (Drenckhahn and Dermietzel, 1988; Bretscher, 1991; Nelson, 2003). Thus, the ability of AE pathogens to dramatically alter the apical cytoskeleton is likely to induce changes in the basolateral and terminal web-associated cytoskeleton including the membrane–cytoskeletal interface. Consistent with this notion, EPEC infection is known to cause the redistribution of many junctional and basolateral domain proteins (Goosney et al., 2001; Muza-Moons et al., 2003, 2004; Guttman et al., 2006, 2007), some of which are recruited to actin pedestals (Peralta-Ramirez et al., 2008; Huett et al., 2009). It will be of interest to determine whether the requirement for calpain in the redistribution of ezrin is reflected in a similar requirement for the redistribution of some or all of these other molecules. Finally, we observed EHEC infection of confluent epithelial cell monolayers resulted in striking cell rounding and "blebbing", which to our knowledge has not been previously reported in response to infection by AE pathogens but is consistent with a general disruption of membrane–cytoskeletal interactions and cell adhesion. Although this damage was calpain-dependent, similar cellular damage was not induced by EPEC, indicating that this manifestation is separable from effacement and is specific to EHEC. This difference could reflect the fact that EHEC encodes a larger number of injected T3SS effectors than does EPEC (Tobe et al., 2006; Wong et al., 2011). Although EHEC-mediated cell rounding could be specifically linked to ezrin cleavage, other actin-associated calpain substrates, such as the focal adhesion protein talin and actin assembly factor and cortactin might also or alternatively be involved (Frame et al., 2002; Franco and Huttenlocher, 2005; Lebart and Benyamin, 2006).

In summary, AE pathogens such as EHEC induce a broad range of cytoskeletal disruption, among them pedestal formation, tight junction disruption and microvillar effacement. These alterations are predicted to diminish both the absorptive capacity and the barrier function of intestinal epithelium, thereby contributing to diarrhea, a major and potentially life-threatening manifestation of disease. The manifold cellular consequences of infection are undoubtedly in part due to diversity of effectors translocated to the cell (Tobe et al., 2006). In addition, an emerging picture is that a central modulator such as calpain, which has the potential to alter many cytoskeletal structures, may be a conduit through which AE family members, as well as other infectious agents, exert pleomorphic pathogenic effects.

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Enhanced actin pedestal formation by enterohemorrhagic *Escherichia coli* O157:H7 adapted to the mammalian host

Michael John Brady[1†], Padhma Radhakrishnan[1†], Hui Liu[1], Loranne Magoun[1], Kenan C. Murphy[1], Jean Mukherjee[2], Arthur Donohue-Rolfe[2], Saul Tzipori[2] and John M. Leong[1,3]*

[1] Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA, USA
[2] Division of Infectious Disease, Department of Biomedical Sciences, Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA, USA
[3] Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA

Edited by:
Elizabeth L. Hartland, The University of Melbourne, Australia

Reviewed by:
Steffen Backert, University College Dublin, Ireland
Michael L. Vasil, University of Colorado Medical School, USA
Charles Martin Dozois, Institut National de la Recherche Scientifique, Canada
Andrew Roe, University of Glasgow, UK

***Correspondence:**
John M. Leong, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA, 02111, USA.
e-mail: john.leong@umassmed.edu

†Michael John Brady and Padhma Radhakrishnan have contributed equally to this work.

Upon intestinal colonization, enterohemorrhagic *Escherichia coli* (EHEC) induces epithelial cells to generate actin "pedestals" beneath bound bacteria, lesions that promote colonization. To induce pedestals, EHEC utilizes a type III secretion system to translocate into the mammalian cell bacterial effectors such as translocated intimin receptor (Tir), which localizes in the mammalian cell membrane and functions as a receptor for the bacterial outer membrane protein intimin. Whereas EHEC triggers efficient pedestal formation during mammalian infection, EHEC cultured *in vitro* induces pedestals on cell monolayers with relatively low efficiency. To determine whether growth within the mammalian host enhances EHEC pedestal formation, we compared *in vitro*-cultivated bacteria with EHEC directly isolated from infected piglets. Mammalian adaptation by EHEC was associated with a dramatic increase in the efficiency of cell attachment and pedestal formation. The amounts of intimin and Tir were significantly higher in host-adapted than in *in vitro*-cultivated bacteria, but increasing intimin or Tir expression, or artificially increasing the level of bacterial attachment to mammalian cells, did not enhance pedestal formation by *in vitro*-cultivated EHEC. Instead, a functional assay suggested that host-adapted EHEC translocate Tir much more efficiently than does *in vitro*-cultivated bacteria. These data suggest that adaptation of EHEC to the mammalian intestine enhances bacterial cell attachment, expression of intimin and Tir, and translocation of effectors that promote actin signaling.

Keywords: host adaptation, actin assembly, translocation, EHEC, intimin, Tir

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is the leading cause of outbreaks of bloody diarrhea and is often associated with the triad of hemorrhagic colitis, thrombocytopenia, and renal failure in the United States (Karmali, 1989). The life threatening sequelae of EHEC infections are due to the production of Shiga toxins (Karmali, 1989; Noel and Boedeker, 1997; Teel et al., 2003). EHEC belongs to a unique subset of intestinal pathogens that cause attaching and effacing (AE) lesions on the intestinal epithelium during infection. AE lesions are histopathological alterations of the intestinal epithelial surface that are characterized by loss of brush border microvilli and formation of actin rich pedestals beneath bound bacteria (Pai et al., 1986). Enteropathogenic *E. coli* (EPEC) is a related intestinal pathogen that causes infantile diarrhea that also generates AE lesions in the host (Frankel et al., 1998; Nataro and Kaper, 1998; Celli et al., 2000; Campellone and Leong, 2003).

An ~35 kB pathogenicity island in *E. coli* O157:H7 termed the locus of enterocyte effacement (LEE) is required for AE lesion formation (McDaniel et al., 1995; McDaniel and Kaper, 1997). Some of the genes on the LEE required for AE lesion formation encode a type III secretion apparatus that injects bacterial effectors into host cells. The best-characterized secreted effector is translocated intimin receptor (Tir) which, when localized in the host cell membrane, serves as a receptor for intimin, a LEE encoded outer membrane adhesin. Intimin is necessary for intimate attachment to epithelial cells and its interaction with Tir is required for production of AE lesions at the enterocyte–bacteria interface. AE lesion formation can be conceptually divided into multiple stages: initial attachment of the bacteria to the host epithelia, type III secretion during which Tir and other *E. coli* secreted proteins (Esps) are translocated into host cells, and finally Intimin-mediated Tir ligation at the plasma membrane, which triggers host cell signaling events that lead to actin assembly (Donnenberg et al., 1997; Hayward et al., 2006; Frankel and Phillips, 2008; Campellone, 2010). Consistent with this model, mammalian cells injected with Esps delivered by a pre-infecting intimin-deficient EPEC mutant are capable of initiating robust actin focusing upon challenge with another strain or particle expressing intimin (Rosenshine et al., 1996; Liu et al., 1999b).

Enterohemorrhagic *Escherichia coli* generates far fewer pedestals than EPEC on cultured mammalian cells. EHEC also exhibits relatively poor mammalian cell binding and actin pedestal formation on cultured monolayers after *in vitro* cultivation, in contrast to the robust AE lesion formation on intestinal epithelia observed during mammalian infection (Karch et al., 1987; Tzipori et al., 1987; Cantey and Moseley, 1991). This apparent paradox raises the possibility that growth in the mammalian host

environment enhances the ability of EHEC to form AE lesions. To directly determine the relative efficiencies of virulence-associated phenotypes, we compared *in vitro*-cultured bacteria to EHEC isolated directly from infected piglets. We found that adaptation of EHEC to the mammalian host is associated with enhanced mammalian cell binding, increased amounts of intimin and Tir, better translocation of functional Tir, and dramatically more efficient pedestal formation on cultured mammalian cells.

MATERIALS AND METHODS

IN VITRO BACTERIAL AND TISSUE CULTURE

The strains and plasmids used in this study are listed (**Table 1**). To enhance biosafety, we and other labs routinely utilized an Stx-deficient derivative of EHEC EDL933 (Riley et al., 1983) when examining virulence features unrelated to toxin production. TUV93-0, lacking Stx, is predicted to be incapable of causing hemorrhagic colitis, but for simplicity, here we nevertheless refer to this as an "EHEC" strain. For infections, EHEC strains were cultured in LB, Miller (BD Difco) broth at 37°C with shaking for approximately 7 h, diluted 1:250 into DMEM with high-glucose (Gibco-BRL) supplemented with 100 mM HEPES (pH 7.4), and incubated at 37°C overnight without shaking (*in vitro*-cultured). It has been shown that there is maximal secretion of Esps by EHEC under these conditions (Ebel et al., 1996). *E. coli* K-12 strains were cultured in LB broth at 37°C with shaking. When appropriate, strains were cultured in media supplemented with 100 μg/mL of ampicillin (pInt or pIL22) or 35 μg/mL of Kanamycin A (pTir).

Table 1 | Strains and plasmids used in this study.

Strain	Description	Designation	Source or reference
TUV93-0	Stx-derivative of EDL933	WT	Donohue-Rolfe et al. (2000)
KC5	EHEC TUV93-0 Δ*tir*	Δ*tir*	Campellone et al. (2002)
KM60	EHEC TUV93-0Δ*eae*	Δ*eae*	Murphy and Campellone (2003)
MC1061	*E. coli* K-12F$^-$Δ(lac)X74*gal*/E *hsd*R *rps*L	K-12	Casadaban and Cohen (1980)
PLASMIDS			
pKC16	pK184-derivative producing EHEC Tir	pTir	Campellone et al. (2002)
pHL6	pUC19-derivative producing EHEC Intimin	pInt	Liu et al. (1999b)
pIL22	pBR322-derivative producing AFA-1	pIL22	Labigne-Roussel et al. (1984)

HEp-2 cells (human laryngeal carcinoma cells) were cultured at 37°C in 5% CO_2 in RPMI-1640 (Gibco-BRL) supplemented with 7% fetal bovine serum and 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2 mM L*-glutamine. HeLa cells (human cervical carcinoma) were cultured at 37°C in 5% CO_2 in DMEM, high-glucose (Gibco-BRL) containing 10% FBS, 2 mM glutamine, and 50 μg/mL penicillin/streptomycin.*

ISOLATION OF EHEC FROM INFECTED GNOTOBIOTIC PIGLETS

Piglets were delivered by cesarean section and maintained in microbiologic isolation (gnotobiotic) for 24 h and infected with an oral challenge $\sim 5 \times 10^9$ EHEC as described previously (Tzipori et al., 1992, 1995). All animals were monitored daily for signs of clinical disease and euthanized 48–72 h after oral challenge.

To harvest bacteria from the intestines, both ileal and spiral colon were collected from each animal and flushed with chilled sterile PBS to remove the majority of gut contents and pooled. To obtain additional adherent EHEC, intestine sections were dissected and the mucosal surfaces were gently scraped with a sterile spatula, flushed with sterile PBS and combined with the initial washings. To separate EHEC from crude intestinal contents, the pooled preparation was centrifuged and washed five to six times with PBS (host-adapted EHEC). To quantitate EHEC density, cells were enumerated using a hemacytometer, re-suspended at 1×10^8 bacteria/mL and confirmed by standard spread plating. The intestinal contents ("mucus" for use in control experiments) from uninfected piglets was obtained similarly.

In one set of control experiments, 20% "mucus" was supplemented to EHEC cultures grown in DMEM with high-glucose to determine whether *in vitro* growth in the presence of intestinal contents altered cell binding or pedestal formation. In a second set of control experiments, 20% "mucus" was added to *in vitro*-cultured EHEC immediately prior to cell binding and pedestal formation assays to determine whether mucus itself promoted mammalian cell binding or pedestal formation. In a third set of control experiments to differentiate between genetic (stable) and phenotypic (transient) adaptation, host-adapted EHEC were passaged *in vitro* as described previously and characterized for cell binding and pedestal formation.

QUANTIFICATION OF BACTERIAL ADHERENCE

Bacterial adherence assays were performed as previously described (Liu et al., 1999a). Briefly, HEp-2 cells were seeded in RPMI-1640 supplemented with 20 mM HEPES (pH 7.0), 2% fetal bovine serum, and 0.5% D-mannose (RHFM medium), infected with approximately 5×10^5 bacteria for an MOI of approximately 10, and incubated for 3 h. Infected monolayers were washed six times in PBS to remove unbound bacteria and lysed with 0.5% TritonX-100. The cell lysates were serially diluted and plated on LB agar to determine the percent of bound bacteria. Significant differences from binding by *in vitro*-cultured EHEC was determined using a two-tailed, paired, Student's *t*-test and defined as a probability of $P \leq 0.05$.

ASSESSMENT OF ACTIN PEDESTAL FORMATION

To quantitate the ability of EHEC strains to form pedestals on mammalian cells, coverslips that had been seeded with HEp-2 cells in RHFM (as described above) were infected with 5×10^5 EHEC. After 3 h incubation at 37°C, the monolayers were washed with PBS and further incubated for 3 h after addition of fresh RHFM. The cells were washed, fixed with 2.5% para-formaldehyde, stained for bacteria with DAPI and filamentous actin with TRITC-conjugated phalloidin (Sigma; Knutton et al., 1989; Liu et al., 1999a, 2002). To quantify pedestal formation, bacteria-associated with actin pedestals was determined as a function of 100 HEp-2

cells examined. One hundred eighty mammalian cells on three coverslips per bacterial preparation were examined in three independent experiments. Significance was determined as previously described (Vingadassalom et al., 2010).

PREPARATION OF CELL LYSATES AND IMMUNOBLOTTING

For Intimin and Tir expression, 10^8 bacteria were re-suspended in 20 μL of 1× Laemmli buffer, lysed by heating to 100°C for 10 min and subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were blocked in PBS + 5% milk (PBSM) before treatment with sheep anti-Intimin (antiserum from a sheep immunized with a C-terminal 300-residues fragment of EHEC 86–24 intimin, gift of A. O'Brien) diluted 1:5000 or rabbit anti-Tir (diluted 1:10,000) antibodies. Following washes, membranes were treated with anti-sheep or anti-rabbit secondary IgG antiserum conjugated to alkaline phosphatase (diluted 1:5000) and developed (Acheson et al., 1995).

For Tir translocation, HEp-2 or HeLa cells were seeded into six well tissue culture plates (Becton–Dickinson Labware) and infected with bacterial strains at an MOI of approximately 10 for 6 h. Following infection, the monolayer were washed with PBS to remove unbound bacteria, lifted with Trypsin, centrifuged for 2 min and the pellet was re-suspended in 1× Laemmli buffer and boiled for 10 min. The samples were analyzed by 10% SDS-PAGE and western blotted for Tubulin and Tir (as described above).

QUANTIFICATION OF SURFACE EXPRESSION OF INTIMIN

An enzyme-linked immunosorbent assay (ELISA) was used to quantify the levels of intimin expression on the bacterial surface (Liu et al., 2002). In brief, equivalent numbers of indicated strains (100 μL aliquots of 1×10^8 bacteria/mL) were seeded on poly-L-lysine-treated 96-well cell culture dishes (Becton–Dickinson Labware), centrifuged for 10 min at $1225 \times g$ and incubated at room temperature for 20 min. The bacteria were then fixed with 3% para-formaldehyde for 1 h at room temperature, washed in PBS and blocked with 5% milk in PBS (PBSM) at room temperature for 1 h. Fixed bacteria were probed with Intimin anti-sera for 1 h at room temperature (diluted 1:500). The plates were washed with PBS and PBS + 0.5% Tween-20, followed by addition of the secondary horseradish peroxidase (HRP)-conjugated anti-sheep IgG antibody (diluted 1:10,000 in PBSM; Sigma). Parallel plates were subjected to anti-O157 antiserum (Difco) diluted 1:1000 in PBSM followed by probing with HRP-conjugated anti-rabbit IgG (Sigma) diluted in PBSM to determine the relative number of bound bacteria. The LPS signal obtained for the different bacterial strains within each experiment were within 5%. The mean OD_{600} values (±SD) of quadruplicate samples after subtraction of background (i.e., signal when the primary antibody was omitted) and normalized to bacterial number are shown.

BACTERIAL BINDING AND PEDESTAL FORMATION IN PRIME AND CHALLENGE ASSAYS

To assess functional Tir delivery, a "prime and challenge" assay was used (Vingadassalom et al., 2010). HEp-2 monolayers were infected with 5×10^5 of either host-adapted or *in vitro*-cultivated EHECΔ*eae* or EHECΔ*tir* with or without plasmid pKC16 (aka pTir) for 3 h, gentamicin (100 μg/ml) treated for 30 min to kill priming bacteria, and challenged with 5×10^5 either native *E. coli* K-12 (vector or un-transformed controls) or *E. coli* K-12 expressing intimin from plasmid pHL6 (aka pInt) and incubated for an additional 3 h at 37°C. To assess bacterial binding, monolayers were lysed and serially plated as described above. To assess actin pedestal formation, infected monolayers were examined microscopically as described above. Significance was determined as previously described (Vingadassalom et al., 2010).

RESULTS

HOST-ADAPTED EHEC BIND AND GENERATE ACTIN PEDESTALS ON CULTURED MAMMALIAN CELLS MORE EFFICIENTLY THAN *IN VITRO*-CULTIVATED EHEC

To test whether EHEC adapts to the mammalian host environment by altering its ability to interact with mammalian cells, we cultivated EHEC O157:H7 strain EDL933 under conditions that have been previously developed to maximize host cell interaction and pedestal formation ("*in vitro*-cultured"; Ebel et al., 1996), or isolated them from the large bowel of gnotobiotic piglets 24–48 h following oral infection ("host-adapted"; see Materials and Methods). Mammalian cell monolayers were infected with either of these two preparations of EHEC and actin pedestal formation was quantified. Whereas less than 5 actin pedestals were observed per 100 mammalian cells for *in vitro*-cultured EHEC, almost 50 pedestals were observed for the same strain after host adaptation (**Figure 1A**). Similar results were observed under identical conditions using HeLa instead of HEp-2 cells (data not shown). This change does not appear to be hereditable because growth of host-adapted bacteria overnight *in vitro* resulted in a complete reversal of enhanced cell attachment activity (data not shown).

Host-adapted EHEC also appeared to bind to mammalian cells much more efficiently than did *in vitro*-cultivated EHEC (**Figure 1A**, "EHEC," compare top two rows). When we quantified viable bacterial counts after lysis of infected monolayers, we found that whereas only 0.2% of *in vitro*-cultivated EHEC bound to monolayers, more than 5% of host-adapted bacteria bound to cells, representing a more than 25-fold increase (**Figure 1B**). Thus, adaptation to the mammalian host results in dramatic increases in both cell attachment and pedestal formation by EHEC.

To determine whether the high efficiency of actin pedestal formation upon host adaptation was solely a consequence of its enhanced ability to bind to monolayers, we required a means to dramatically increase mammalian cell binding of *in vitro*-cultured EHEC. Accordingly, we ectopically expressed the afimbrial adhesion AFA-1 from uropathogenic *E. coli*, encoded on pIL22 to artificially increased mammalian cell binding (Labigne-Roussel et al., 1984). Indeed, AFA-1 increased cell attachment by *in vitro*-cultivated EHEC to a level comparable to that observed for host-adapted EHEC (**Figure 1B**). Nevertheless, this enhanced cell binding was not associated with a significant increase in the frequency of actin pedestals (**Figure 1A**). Thus, the higher efficiency of pedestal formation by host-adapted EHEC is not simply a consequence of increased cell binding, suggesting that host adaptation results in expression of EHEC products that specifically contribute to actin signaling.

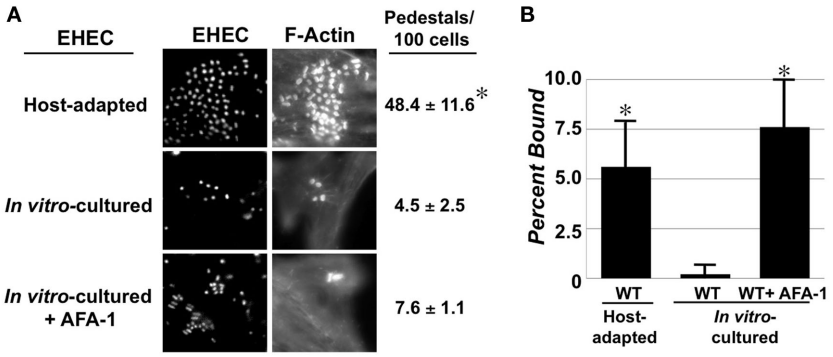

FIGURE 1 | Enterohemorrhagic *Escherichia coli* actin pedestal formation and adhesion are enhanced upon host adaptation. (A) Mammalian cell monolayers were infected with host-adapted EHEC (top), *in vitro*-cultured EHEC (middle) or *in vitro*-cultured EHEC ectopically expressing the afimbrial adhesin AFA-1 (bottom). Bacteria were stained with DAPI ("EHEC") and actin pedestals with Alexa568-phalloidin ("F-actin"). The percentage of actin pedestals (±SD) per 100 mammalian cells was determined visually (right). **(B)** Binding of the same three strains to mammalian cell monolayers was determined by plating for viable counts. Data represent the mean ± SD from three independent experiments. $*P \leq 0.05$.

INTIMIN EXPRESSION IS INCREASED DURING HOST ADAPTATION BUT IS NOT SOLELY RESPONSIBLE FOR ENHANCED PEDESTAL FORMATION

Given that intimin participates in cell attachment and pedestal formation, we assessed the relative levels of this adhesin expressed by *in vitro*-cultivated and host-adapted EHEC by Western blotting. As expected, no intimin was detected in extracts of *in vitro*-cultivated EHECΔ*eae* (**Figure 2A**, rightmost lane). Greater amounts of intimin were produced by host-adapted than by *in vitro*-cultivated EHEC (**Figure 2A**, first two lanes). Densitometric analysis suggested a fourfold increase, but was complicated by the multitude of intimin-related species that are typically produced (data not shown; Donnenberg et al., 1993; McKee and O'Brien, 1996; Agin and Wolf, 1997; Liu et al., 2002). The apparent increase in expression of intimin in whole cell lysates by host-adapted bacteria was reflected in the level of surface protein detected by ELISA of intact bacteria using anti-intimin antiserum, which revealed that host-adapted bacteria expressed more than threefold higher levels of the adhesin on their surface than their *in vitro*-cultivated counterparts (**Figure 2B**).

In addition to promoting pedestal formation by binding to Tir, intimin may enhance binding by recognizing mammalian proteins, such as β1-chain integrins (Frankel et al., 1996) and nucleolin (Sinclair and O'Brien, 2002). To determine whether intimin overexpression by host-adapted EHEC can account for the observed high efficiency of actin pedestal formation, we artificially increased intimin expression of *in vitro*-cultured EHEC by introducing a plasmid ("pInt") that encodes intimin. When evaluated by immunoblotting for total intimin and ELISA for surface expression, EHEC harboring pInt expressed levels of intimin comparable to those achieved upon host adaptation (**Figures 2A,B**, middle lanes), while EHEC harboring a control vector expressed levels of intimin comparable to *in vitro*-cultured WT (data not shown). However, the overexpression of intimin by *in vitro*-cultivated EHEC did not result in a concomitant increase in either host cell binding or actin pedestal formation (**Figure 2C**).

To determine whether the increased production of intimin by host-adapted bacteria is responsible the enhanced mammalian binding we observed, we tested cell binding of an EHEC *eae* mutant, which cannot produce intimin. We found that although host-adapted EHEC *eae* was, as predicted, completely incapable of generating actin pedestals (**Figure 2C**, right), this strain bound to mammalian cells indistinguishably from host-adapted wild type EHEC. Thus, the high level of mammalian cell binding observed for host-adapted EHEC does not reflect intimin-mediated attachment. Clearly, other bacterial factors are required to account for the high efficiency of cell interaction observed upon host adaptation of EHEC.

Tir EXPRESSION IS INCREASED DURING HOST ADAPTATION BUT IS NOT SOLELY RESPONSIBLE FOR ENHANCED PEDESTAL FORMATION

Translocated intimin receptor and intimin are expressed from a co-transcribed mRNA (Sanchez-SanMartin et al., 2001) so we also compared Tir expression by host-adapted or *in vitro*-cultivated bacteria. Immunoblot analysis of cell lysates revealed that an ∼80 kDa species that Tir production by host-adapted bacteria was considerably greater than by *in vitro*-cultured EHEC (**Figure 3A**, lanes 1 and 2); densitometric analyses suggested an approximately fivefold increase (data not shown).

To determine if the increase in Tir expression was responsible for the enhanced adherence and pedestal formation by host-adapted bacteria, we introduced a high-copy plasmid expressing Tir (pTir) into EHEC that resulted in the levels of Tir expression comparable to that of host-adapted EHEC (**Figure 3A**, lane 3). Consistent with the observation that adherence to monolayers was not influenced by the absence or overexpression of intimin (**Figure 2**), the absence or overexpression of Tir also had no effect on binding of *in vitro*-cultivated EHEC to mammalian cells (**Figure 3B**). In addition, overexpression of Tir by *in vitro*-cultivated EHEC did not result in an increase in pedestal formation (**Figure 3B**, right). These data indicate that although Tir production is increased upon host adaptation, this increase is not sufficient to account for the dramatic increase in the ability to generate actin pedestals upon growth in the mammalian host.

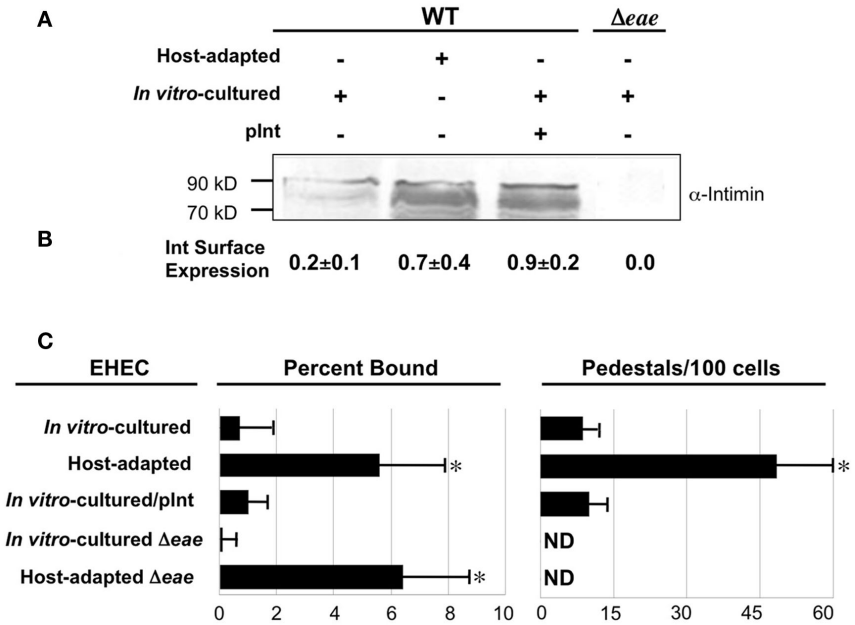

FIGURE 2 | Intimin expression is increased during host adaptation but is not solely responsible for enhanced pedestal formation. (A) Equivalent numbers of bacteria were utilized to generate bacterial lysates of host-adapted or *in vitro*-cultivated EHEC that were subjected to immunoblotting using anti-intimin antibody. The *in vitro*-cultivated EHEC included wild type bacteria ±pInt, a plasmid that encodes intimin (pInt, lane 3), and EHEC Δ*eae*, which does not express intimin (lane 4). Molecular weight markers are indicated at left. **(B)** Equivalent numbers of the indicated strains were added to microtiter wells, fixed, and the relative number of bound bacteria was normalized by ELISA using anti-O157 antiserum. Parallel microtiter wells were subjected to anti-intimin ELISA to determine relative intimin surface expression. Shown are the mean OD_{600} values (±SD) of quadruplicate samples after subtraction of background (i.e., signal when the primary antibody was omitted) and normalized to bacterial number. **(C)** The percent of bacteria bound, determined by plating for viable counts, and the frequency of pedestal formation was determined as described in **Figure 1**. ND, not detected. $*P \leq 0.05$.

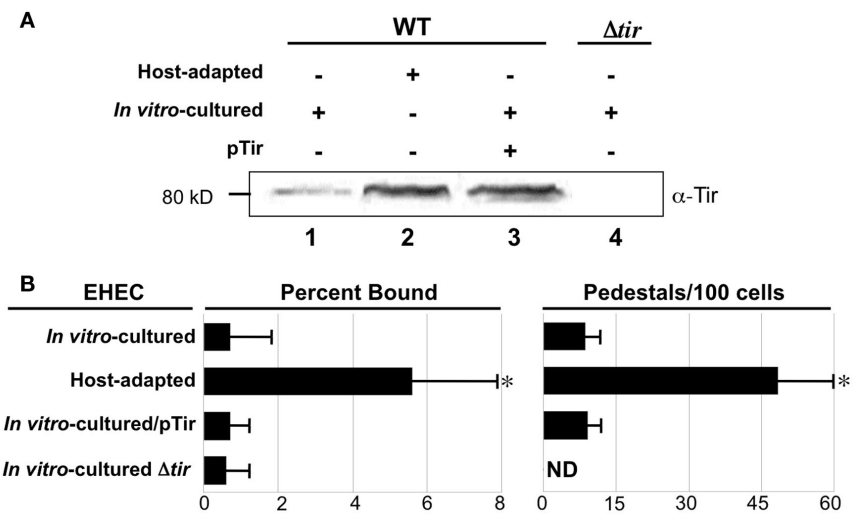

FIGURE 3 | Translocated intimin receptor expression is increased during host adaptation but is not solely responsible for enhanced pedestal formation. **(A)** Equivalent numbers of bacteria were utilized to generate bacterial lysates of host-adapted or *in vitro*-cultivated EHEC that were subjected to immunoblotting using anti-intimin antibody. The *in vitro*-cultivated EHEC included wild type bacteria ± pTir a plasmid that encodes Tir (pTir, lane 3), and EHEC Δ *tir* (lane 4). Molecular weight is indicated at left. **(B)** Percent bacteria bound and the frequency of pedestal formation was determined as described in **Figure 1**. ND, not detected. $*P \leq 0.05$.

HOST ADAPTATION RESULTS IN INCREASED Tir TRANSLOCATION

Tir functions to promote cell attachment and pedestal formation only after insertion into the mammalian cell plasma membrane

After translocation into the mammalian host, EHEC Tir becomes serine phosphorylated by PKA, a modification that can be detected by an increase in apparent molecular mass upon SDS-PAGE (DeVinney et al., 1999; Warawa and Kenny, 2001). To assess the relative Tir translocation efficiencies, we infected HeLa cell monolayers with equivalent numbers of either host-adapted or *in vitro*-cultivated EHEC, and characterized the amount of modified Tir by Western blotting (Kenny, 1999). Analysis of replicate samples revealed that host-adapted EHEC translocates a significantly greater amount of Tir into monolayers than *in vitro* grown EHEC (**Figure 4**, compare lanes 1 and 2 with lanes 3 and 4). Similar results were observed under identical conditions using HeLa instead of HEp-2 cells (data not shown). Enhanced Tir translocation by host-adapted EHEC is not a simple consequence of enhanced host cell binding, because an increase in modified Tir was not observed when HeLa cells were infected with *in vitro*-cultivated EHEC that is highly adhesive by virtue of expressing AFA-1 (**Figure 4**, lane 5). Importantly, since there is approximately equivalent amounts of modified Tir, Tir translocation appears to be independent of AFA-1-mediated enhanced cell binding, suggesting that ectopic expression of AFA-1 neither increased nor decreased the efficiency of type III secretion (**Figure 4**, compare lanes 4 and 5).

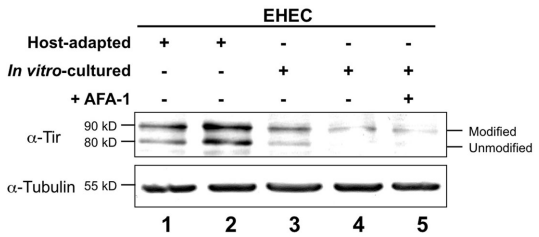

FIGURE 4 | Translocated intimin receptor translocation is enhanced upon host adaptation. Mammalian cell monolayers were infected for 6 h with equivalent numbers of host-adapted EHEC isolated from the intestine of two different gnotobiotic piglets (lanes 1–2) or *in vitro*-cultured EHEC strains alone (lanes 3–4) or *in vitro*-cultured EHEC harboring a plasmid encoding AFA-1 (lane 5). Lysates of infected monolayers were resolved by SDS-PAGE and immunoblotted for Tir and tubulin. Western blots from a representative experiment are shown. The migration of unmodified and modified Tir is indicated (right), and numbers on the left indicate apparent molecular mass.

To determine if the apparent increased translocation of Tir by host-adapted bacteria resulted in enhanced Tir function, we utilized a "prime and challenge" assay

We first infected monolayers with either host-adapted or *in vitro*-cultured EHECΔ*eae*, which translocates Tir but is not capable of intimin-mediated binding. Bacteria were removed by gentamicin treatment and washing. These "primed" monolayers were then "challenged" with either an *E. coli* K-12 strain ("K-12") or an *E. coli* strain harboring a plasmid expressing EHEC intimin ("K-12/pInt"), and after washing, bound bacteria were quantified by plating for viable counts.

Binding of *E. coli* K-12/pInt to unprimed mammalian cell monolayers (data not shown), or monolayers primed with *in vitro*-cultured EHECΔ*tir*, was indistinguishable from binding of *E. coli* K-12 (i.e., ~1% of inoculum), indicating that in this experimental system, intimin does not promote binding to an endogenous host cell receptor (**Table 2**, top row). *E. coli* K-12/pInt also did not demonstrate significant binding to monolayers primed with *in vitro*-cultured EHECΔ*eae*, indicating that, upon *in vitro* growth, EHEC does not have the ability to translocate sufficient levels of Tir to allow detection in this functional assay (**Table 2**, row 2). Artificial overexpression of Tir in this strain, by introduction of pTir, was also not sufficient to promote translocation of functional amounts of Tir (**Table 2**, row 3). In contrast, *E. coli* K-12/pInt bound at levels 10-fold above background to monolayers that had been primed with host-adapted EHECΔ*eae* (**Table 2**, bottom row). Microscopic visualization of the monolayers after staining bound bacteria and F-actin revealed that *E. coli* K-12/pInt also generated pedestals on these cells, but not on cells primed with *in vitro*-cultured EHECΔ*eae*. These data indicate that growth in the mammalian host results in enhanced levels of translocation.

DISCUSSION

Bacteria are capable of remarkable phenotypic adaptation during mammalian infection and several bacterial pathogens, including the AE pathogen *C. rodentium*, exhibit enhanced infectivity upon

Table 2 | Translocation of functional Tir is enhanced upon host adaptation.

Priming strain		Challenge strain			
EHEC	Plasmid	K-12		K-12/pInt	
		Percent bound	Pedestals/100 cells	Percent bound	Pedestals/100 cells
Host-adapted EHECΔ*eae*	None	0.8 ± 0.1	ND	14.0 ± 3.2*	12.6 ± 1.5*
In vitro-cultured EHECΔ*eae*	None	0.9 ± 0.3	ND	1.5 ± 0.7	0.0 ± 0.0
In vitro-cultured EHECΔ*tir*	None	0.7 ± 0.5	ND	1.0 ± 0.5	0.0 ± 0.0
In vitro-cultured EHECΔ*eae*	pTir	ND	ND	1.3 ± 0.7	0.0 ± 0.0

*Monolayers were preinfected (or "primed") with host-adapted EHEC, in vitro-cultured EHEC, or in vitro-cultured EHEC over expressing Tir. Pre-infecting bacteria were removed and primed monolayers were infected ("challenged") with E. coli K-12/pInt. Bacterial attachment and actin pedestal formation were determined as described above, and shown are the mean ± SD from three independent experiments. ND, not determined. *$P \leq 0.05$.*

passage through mammalian hosts (Merrell et al., 2002; Wiles et al., 2005). We hypothesized that host adaptation by EHEC might explain the longstanding paradox that *in vitro*-cultured EHEC typically attach to and generate actin pedestals on mammalian cell monolayers with low efficiency (Karch et al., 1987; Cantey and Moseley, 1991) yet generate extensive AE lesions on intestinal epithelial cells during mammalian infection (Tzipori et al., 1987). In fact, we found that EHEC isolated from the piglet intestine bound to HEp-2 cell monolayers ∼25-fold more efficiently and generated actin pedestals with ∼10-fold more efficiently than *in vitro*-cultivated bacteria. AE lesion formation is associated with enhanced colonization (Vlisidou et al., 2006; Ritchie et al., 2008; Crepin et al., 2010), and it is tempting to speculate that the increased efficiency of interaction with cultured mammalian cells observed upon host adaptation is associated with the increased colonization efficiency. Indeed, the hyperinfectious phenotype attained by *C. rodentium* following passage through mice is associated with greater host cell binding and pedestal formation, similar to that observed in this study (Wiles et al., 2005; Bishop et al., 2007).

Host-adapted bacteria produced significantly higher levels of intimin and Tir, two proteins documented to promote both bacterial adhesion and localized actin assembly (Jerse et al., 1990; Kenny et al., 1997). However, the enhanced cell attachment phenotype by host-adapted EHEC did not require intimin expression, and this intimin-independence suggests that Tir is also unlikely to be required for this phenotype. In addition, artificially increasing levels of these proteins by *in vitro*-cultivated bacteria was not sufficient to phenocopy host-adapted EHEC. Several EHEC surface proteins have been demonstrated or postulated to promote attachment to mammalian cells (i.e., AidA, Iha, Lpf, ToxB, HCP; Ebel et al., 1998; Spears et al., 2006; Xicohtencatl-Cortes et al., 2009; Yin et al., 2009a,b, 2011), but the role of any of these adhesins in binding of host-adapted EHEC remains to be demonstrated.

The high efficiency of cell attachment and pedestal formation by host-adapted EHEC appeared to be at least in part a manifestation of enhanced type III translocation. Immunoblotting of extracts of infected monolayers suggested that the translocated effector Tir was delivered more efficiently to mammalian cells by host-adapted than by *in vitro*-cultivated EHEC. In addition, in an assessment of Tir translocation that depends on the detection of two critical Tir functions, we found that preinfection of monolayers with host-adapted but not *in vitro*-cultured bacteria resulted in significantly greater Tir-mediated bacterial binding and actin pedestal formation. Given that actin pedestal formation is largely dependent on a second translocated effector, $EspF_U$ (TccP; Campellone et al., 2004; Garmendia et al., 2004) it appears likely that translocation of at least some other type III effectors is also increased during host adaptation.

The AE pathogens EPEC and *C. rodentium* each encode a type IV pilus capable of retraction that is required for colonization (Donnenberg et al., 1992; Mundy et al., 2003), and the ability of the EPEC type IV pilus to retract has recently been linked to increased type III translocation and actin pedestal formation (Zahavi et al., 2011). Thus, it is tempting to speculate that the EHEC retractable type IV pilus HCP, which appears to be expressed during human infection (Xicohtencatl-Cortes et al., 2007), may play a role in promoting cell attachment and type III translocation by host-adapted EHEC. We showed here that artificial enhancement of bacterial binding by the ectopic expression of the afimbrial adhesin AFA-1, which is not capable of retraction, did not result in a concomitant increase in Tir translocation or pedestal formation by *in vitro*-cultured EHEC. Although AFA-1 is not normally expressed in EHEC, it does not appear to interfere with (or enhance) Tir translocation and importantly, and its ectopic expression allowed us to test whether increased pedestal formation was solely a function of increased (albeit artificial) adherence. We cannot exclude the possibility that host-adapted bacteria express one or more adhesin(s) that, by co-ordinated regulation or function with the type III system, facilitate(s) both cell binding and subsequent Tir translocation. Studies to identify the bacterial factor(s) responsible for enhanced cell attachment and pedestal formation by EHEC are ongoing.

Enterohemorrhagic *Escherichia coli dam* and *hfq* mutants, which each suffer pleomorphic regulatory alterations (Murphy et al., 2008; Bhatt et al., 2011), exhibit increased cell binding, effector translocation, and pedestal formation, similar to that observed here for host-adapted EHEC (Campellone et al., 2007; Hansen and Kaper, 2009; Shakhnovich et al., 2009). It is possible that loss of *dam* or *hfq* function mimics some aspects of host adaptation. The induction of cell attachment and pedestal forming capacities by host-adapted EHEC were lost upon overnight *in vitro* culture (P. Radhakrishnan, M. J. Brady, data not shown), indicating that these phenotypes are due to a reversible alteration by EHEC growing in the host, similar to the transient nature of enhanced host cell interaction by *C. rodentium* (Bishop et al., 2007). Although the regulation of the LEE pathogenicity island that encodes the type III translocation system of AE pathogens has been studied extensively (Ando et al., 2007; Musken et al., 2008; House et al., 2009; for review, see Mellies et al., 2007), it is not known what environmental signal(s) results in host adaptation by EHEC. Quorum sensing and growth phase have been shown to regulate virulence factors of AE or other intestinal pathogens (Puente et al., 1996; Sperandio et al., 1999; Antunes et al., 2010). However, manipulation of the growth phase of *in vitro*-cultivated EHEC did not result in efficient host cell attachment or actin pedestal formation (P. Radhakrishnan, unpublished observations) and broth cultures of EHEC that achieved approximately the same density of bacteria as was attained in the piglet intestine ($\sim 10^9$ bacteria/ml) bound to and generated pedestals on mammalian cell monolayers inefficiently, indicating that quorum sensing *per se* is not a sufficient trigger for host adaptation in binding and type III translocation. In addition, mammalian factors such as catecholamines have been implicated in the regulation of EHEC virulence, but *in vitro* culture of EHEC in the presence of epinephrine or porcine intestinal mucus did not increase mammalian cell binding or pedestal formation (M. J. Brady and P. Radhakrishnan, unpublished observation). Thus, future studies are required to understand the regulatory pathway(s) responsible for host adaptation by EHEC. Such investigations, as well as studies to identify the microbial products that promote enhanced cell interaction of bacteria growing within the mammalian host, would provide both insight into the pathogenesis of EHEC infection and potential targets for therapeutic intervention.

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Allele- and Tir-independent functions of intimin in diverse animal infection models

Emily M. Mallick[1], Michael J. Brady[2], Steven A. Luperchio[3], Vijay K. Vanguri[4], Loranne Magoun[2], Hui Liu[2], Barbara J. Sheppard[3], Jean Mukherjee[5], Art Donohue-Rolfe[5], Saul Tzipori[5], John M. Leong[1,2]* and David B. Schauer[3,6†]

[1] *Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA, USA*
[2] *Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, USA*
[3] *Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA*
[4] *Department of Pathology, University of Massachusetts Medical School, Worcester, MA, USA*
[5] *Division of Infectious Diseases, Tufts University School of Veterinary Medicine, North Grafton, MA, USA*
[6] *Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA, USA*

Edited by:
Elizabeth L. Hartland, The University of Melbourne, Australia

Reviewed by:
Olivier Marchès, Queen Mary University of London, UK
Alan Phillips, Institute of Child Health, UK

****Correspondence:***
John M. Leong, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA.
e-mail: john.leong@tufts.edu

[†] *Deceased*

Upon binding to intestinal epithelial cells, enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *E. coli* (EPEC), and *Citrobacter rodentium* trigger formation of actin pedestals beneath bound bacteria. Pedestal formation has been associated with enhanced colonization, and requires intimin, an adhesin that binds to the bacterial effector translocated intimin receptor (Tir), which is translocated to the host cell membrane and promotes bacterial adherence and pedestal formation. Intimin has been suggested to also promote cell adhesion by binding one or more host receptors, and allelic differences in intimin have been associated with differences in tissue and host specificity. We assessed the function of EHEC, EPEC, or *C. rodentium* intimin, or a set of intimin derivatives with varying Tir-binding abilities in animal models of infection. We found that EPEC and EHEC intimin were functionally indistinguishable during infection of gnotobiotic piglets by EHEC, and that EPEC, EHEC, and *C. rodentium* intimin were functionally indistinguishable during infection of C57BL/6 mice by *C. rodentium*. A derivative of EHEC intimin that bound Tir but did not promote robust pedestal formation on cultured cells was unable to promote *C. rodentium* colonization of conventional mice, indicating that the ability to trigger actin assembly, not simply to bind Tir, is required for intimin-mediated intestinal colonization. Interestingly, streptomycin pre-treatment of mice eliminated the requirement for Tir but not intimin during colonization, and intimin derivatives that were defective in Tir-binding still promoted colonization of these mice. These results indicate that EPEC, EHEC, and *C. rodentium* intimin are functionally interchangeable during infection of gnotobiotic piglets or conventional C57BL/6 mice, and that whereas the ability to trigger Tir-mediated pedestal formation is essential for colonization of conventional mice, intimin provides a Tir-independent activity during colonization of streptomycin pre-treated mice.

Keywords: ***Citrobacter rodentium*, intimin, enterohemorrhagic *Escherichia coli*, invasin, enteropathogenic *Escherichia coli***

INTRODUCTION

The family of attaching and effacing (AE) pathogens consists of enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *E. coli* (EPEC), and *Citrobacter rodentium*. EHEC colonizes the large intestine and can result in diarrhea, hemorrhagic colitis, and life-threatening hemolytic uremic syndrome (Kaper et al., 2004; Pennington, 2010). The highly related EPEC colonizes the small intestine and is a causative agent of infantile diarrhea in the developing world (Chen and Frankel, 2005; Spears et al., 2006). *C. rodentium*, is a related murine pathogen that typically colonizes the large intestine and causes transmissible murine colonic hyperplasia, characterized by colonic epithelial cell proliferation and high rates of mortality in suckling animals (reviewed in Luperchio and Schauer, 2001; Mundy et al., 2005).

The three pathogens are so-named AE pathogens because they each colonize the intestinal epithelium by inducing in host cells "AE lesions," which consist of effacement of brush border microvilli, intimate adherence of bacteria, and polymerization of actin into a pedestal-like extension of the epithelial cell beneath the bound bacterium (Moon et al., 1983; for review, see Kaper et al., 2004). Bacteria entirely incapable of generating AE lesions are severely defective for colonization and disease (Donnenberg et al., 1993a,b; Schauer and Falkow, 1993b; Tzipori et al., 1995; Deng et al., 2003; Ritchie et al., 2003), while bacteria still capable of intimate attachment but defective selectively for pedestal formation are moderately attenuated (Ritchie et al., 2008; Crepin et al., 2010).

The ability to generate the AE phenotype by these organisms requires the locus of enterocyte effacement (LEE), a pathogenicity

island that encodes a type III secretion system (TTSS) and several translocated effectors (Kenny and Finlay, 1995; McDaniel et al., 1995). Translocated intimin receptor (Tir), a type III translocated effector critical for intimate bacterial attachment and actin pedestal formation, becomes localized in the host plasma membrane with the N- and C-terminal cytoplasmic domains (for review, see Lommel et al., 2004; Frankel and Phillips, 2008; Campellone, 2010). The central, extracellular domain is recognized by the outer membrane adhesin intimin, encoded by the *eae* gene (Jerse et al., 1990; Donnenberg and Kaper, 1991). The intimin N-terminus promotes outer membrane localization and multimerization, whereas the intimin C-terminus encodes its adhesive activities (Frankel et al., 1995; Liu et al., 1999; Batchelor et al., 2000; Luo et al., 2000; Yi et al., 2010).

It has been postulated that in addition to binding Tir, intimin possesses host receptor adhesive activities that also contribute to colonization. For instance, the intimin related *Yersinia pseudotuberculosis* invasin protein binds to β_1-chain integrins (Isberg et al., 1987), and EPEC intimin was shown to be capable of recognizing β_1-chain integrins, albeit with apparently much lower affinity (Frankel et al., 1996a). Nucleolin is recognized by EHEC intimin (Sinclair and O'Brien, 2002) and localized beneath cell-associated EPEC during infection of cultured monolayers (Dean and Kenny, 2011). Finally, intimin but not Tir, contributes to the disruption of epithelial barrier function (Dean and Kenny, 2004), suggesting the existence of Tir-independent functions of intimin.

Although EPEC and EHEC intimin have been demonstrated to be interchangeable for pedestal formation on cultured cells, intimin exhibits considerable allelic variation in the C-terminal domain responsible for adhesive activity (Frankel et al., 1994), and the intimin alleles from the canonical EHEC, EPEC, and *C. rodentium* strains are distinct and have been associated with differences in function. For example, although tissue tropism during infection of human intestinal explants is multifactorial, infection of intestinal tissue *ex vivo* suggests that intimin of EHEC O157:H7 (also known as intimin γ) promotes colonization of different epithelial types than intimin of canonical EPEC (intimin α) or *C. rodentium* (intimin β) (Phillips and Frankel, 2000; Fitzhenry et al., 2002; Girard et al., 2005; Mundy et al., 2007). In addition, whereas wild type EHEC colonizes the large bowel of gnotobiotic piglets, an EHEC strain harboring a plasmid expressing EPEC intimin acquired the additional ability to colonize the small intestine (Tzipori et al., 1995). Allelic differences may also contribute to differences in species host range, because whereas *C. rodentium* expressing EPEC intimin is able to efficiently colonize Swiss NIH and C3H/HeJ mice (Schauer and Falkow, 1993b; Frankel et al., 1996b; Hartland et al., 2000), *C. rodentium* expressing a derivative EPEC intimin harboring the adhesive domain of EHEC intimin provided only poor colonization function in these animals (Hartland et al., 2000; Mundy et al., 2007).

To gain insight into the critical activities of intimin, we assessed the *in vivo* functionality of EHEC, EPEC, or *C. rodentium* intimin, or a set of EHEC intimin derivatives with varying Tir-binding abilities. Colonization was not detectably altered by allele-specific differences in intimin in two animal infection models. Notably, whereas the ability to trigger Tir-mediated pedestal formation was found to be essential for colonization of conventional mice, intimin provided a Tir-independent function during colonization of antibiotic pre-treated mice.

MATERIALS AND METHODS

MEDIA, BACTERIAL STRAINS, AND GROWTH CONDITIONS

Bacteria were stored in Luria–Bertani (LB) broth (American Bioanalytical, Natick, MA, USA) with 50% glycerol at either −80° or −135°C. Bacteria were grown at 37°C in LB broth, in Antibiotic Medium 3 (Difco, Laboratories, Detroit, MI, USA), on LB agar (American Bioanalytical, Natick, MA, USA), on MacConkey lactose agar, or on eosin–methylene blue agar (Difco Laboratories, Lawrence, KS, USA). Where indicated, kanamycin, chloramphenicol, gentamicin, ampicillin, and zeocin were added at final concentrations of 20, 10, 100, 100 or 750, and 75 μg/ml respectively. The bacterial strains and plasmids used in this study are listed in **Tables 1** and **2**.

CONSTRUCTION OF pCVD438 (pINT$_{EPEC}$) DERIVATIVES

Genomic DNA from EHEC O157:H7 strain EDL933 and plasmid pInt$_{EPEC}$ were purified using standard methodologies. All primers used to construct these pCVD438 derivatives are listed in **Table 3**. To construct pHL69 (pInt$_{EHEC}$), a two-step amplification (fusion PCR) was performed. First, the *Pfu* Turbo PCR system (Stratagene, La Jolla, CA, USA) was used to generate three fragments: the region 5′ of the *eae*$_{EPEC}$ open reading frame (ORF) in pInt$_{EPEC}$ (from the *Hin*dIII site to the *eae* start codon), the region 3′ of the *eae*$_{EPEC}$ ORF in pInt$_{EPEC}$ (from the *eae* stop codon to the *Sal*I site), and the coding region of the *eae*$_{EHEC}$ gene from EDL933. To generate the first two fragments, pInt$_{EPEC}$ was restriction enzyme digested with *Bst*EII to delete an internal 1,847-bp fragment (nucleotides 585–2432 of *eae*$_{EPEC}$) and used as the PCR template. The coding region of EDL933 *eae*$_{EHEC}$ was amplified from genomic DNA. Each primer at a junction point in the fusion PCR was tailed with a 9- to 15-bp overhanging sequence from the neighboring fragment. The amplification products were isolated using the QIAquick PCR purification kit (QIAgen Inc., Valencia, CA, USA), mixed, and subjected to a second round of amplification with the pACYC184 primers listed in **Table 3**. The fusion PCR product was confirmed by DNA sequencing and ligated into the corresponding *Hin*dIII/*Sal*I site of pInt$_{EPEC}$, effectively replacing the *eae*$_{EPEC}$ ORF of pInt$_{EPEC}$ with that of EHEC EDL933. The ligation mix was introduced into laboratory strains of *E. coli* by high-voltage electroporation and chloramphenicol-resistant clones were isolated.

A similar methodology was used to generate pInv, pInv–Int395, pInv–Int181, and pInv–Int100 (**Table 2**). In the case of pInv, the coding region of the *Y. pseudotuberculosis inv* gene was amplified from pRI203. For the latter three plasmids, the hybrid invasin–intimin alleles had been previously generated and cloned into pT7-4 (Liu et al., 1999). These pT7-4 derivatives, pHL35, pHL49, and pHL55, respectively, were used as template DNA to amplify the coding region for pInv–Int395, pInv–Int181, and pInv–Int100, respectively.

GENERATION OF *C. RODENTIUM* STRAINS

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAgen, Valencia, CA, USA) and quantified by UV spectrophotometry. Plasmids were introduced into the *C. rodentium*Δ*eae*

Table 1 | Bacterial strains used in this study.

Strain	Description or genotype	Source or reference
DBS100	*C. rodentium* (prototype TMCH isolate, ATCC 51459, original biotype 4280)	Barthold et al. (1976), Schauer and Falkow (1993b)
DBS255	*C. rodentium*Δ*eae*; Kan^R	Schauer and Falkow (1993a)
DBS434	*C. rodentium*Δ*eae*/pCVD438	Frankel et al. (1996b)
SAL31	*C. rodentium*Δ*eae*/pHL69	This study
SAL32	*C. rodentium*Δ*eae*/pHL70	This study
SAL56	*C. rodentium*Δ*eae*/pHL76	This study
SAL57	*C. rodentium*Δ*eae*/pHL79	This study
SAL58	*C. rodentium*Δ*eae*/pHL80	This study
DH5α	F-ø80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (r_K^- m_K^+) *deo*R *thi*-1 *pho*A *sup*E44 I^{-gyr}A96 *rel*A1 *gal*$^-$	Gibco BRL
SAL35	DH5α/pHL70F-ø80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (r_K^- m_K^+) *deo*R *thi*-1 *pho*A *sup*E44 I^{-gyr}A96 *rel*A1 *gal*$^-$	This study
EDL933	EHEC O157:H7	Riley et al. (1983)
JPN15.96	EPEC *eae*::Tn*phoA*	Jerse et al. (1990)
BL21 DE3/pDV205	BL21 DE3 containing EHEC *tir* expression vector	Vingadassalom et al. (2009)
EDL933	EHEC O157:H7 prototype	Riley et al. (1983)
TUV93-0	Stx^- derivative of EDL933	Campellone et al. (2002)
KM60	EHEC TUV93-0 Δ*eae*	Murphy and Campellone (2003)
KM48	EHEC TUV93-0 Δ*eae::cat-sacB*	Murphy and Campellone (2003)
JPN15/pMAR7	Amp^R derivative of EPEC E2348/69 O127:H6 prototype	Jerse et al. (1990)
LM-1	KM46 derivative expressing EHEC *tir-cesT* and EPEC *eae*	This study
C. rodentium Δ*tir*	DBS100 with an in-frame deletion of *tir*; Zeo^R (i.e., zeocin resistance)	This study

Table 2 | Description of plasmids used in study.

Plasmid	Protein	Description	Reference
pCVD438	Int_{EPEC}	pACYC184-derived plasmid encoding *eae* from E2348/69	Donnenberg and Kaper (1991)
pHL69	Int_{EHEC}	pCVD438 with eae_{EPEC} ORF replaced by eae_{EHEC} ORF	This study
pHL70	Inv	pCVD438 with eae_{EPEC} ORF replaced by *inv* ORF	This study
pHL76	Inv–Int395	pCVD438 with eae_{EPEC} ORF replaced by the 5′ 489 codons of *inv* fused to the 3′ 395 codons of eae_{EHEC}.	This study
pHL79	Inv–Int181	pCVD438 with eae_{EPEC} ORF replaced by the 5′ 793 codons of *inv* fused to the 3′ 181 codons of eae_{EHEC}	This study
pHL80	Inv–Int100	pCVD438 with eae_{EPEC} ORF replaced by the 5′ 878 codons of *inv* fused to the 3′ 100 codons of eae_{EHEC}	This study
pT7-4		Amp^R $P_{\phi 10}$	Tabor and Richardson (1985)
pRI203		pT7-4 with *inv* from *Y. pseudotuberculosis*	Isberg et al. (1987)
pACYC184		cloning vector, Cam^R, Tet^R	Rose (1988)
pHL35		pT7-4 with *inv* (bp 1–1467)/eae_{EHEC} (bp 1617–2802); produces the protein Inv–Int395	Liu et al. (1999)
pHL49		pT7-4 with *inv* (bp 1–2379)/eae_{EHEC} (bp 2260–2802); produces the protein Inv–Int181	Liu et al. (1999)
pHL55		pT7-4 with *inv* (bp 1–2634)/eae_{EHEC} (bp 2503–2802); produces the protein Inv–Int100	Liu et al. (1999)
pDV205	EHEC Tir	pET21 containing EHEC *tir*	Vingadassalom et al. (2009)
pUC19		High copy cloning vector, Amp^R	Yanisch-Perron et al. (1985)
pHL4	Int_{EPEC}	pUC19 derivative encoding EPEC *eae*	Liu et al. (1999)
pHL6	Int_{EHEC}	pUC19 derivative encoding EHEC *eae*	Liu et al. (1999)
pTP223		Produces λ-gam-bet-exa downstream of lac promoter	Murphy and Campellone (2003)
pKD46		Expresses lambda-red recombinase, Amp^R	Datsenko and Wanner (2000)

strain by chemical transformation using calcium chloride. Successful transformants were selected with chloramphenicol and were confirmed to be carrying the proper plasmid.

GENERATION OF AN EHEC STRAIN ENCODING EPEC INTIMIN

A three-way PCR was performed using templates encoding EHEC *cesT* with 3′ tail encoding EPEC *eae* (∼750 bp), EPEC

Table 3 | DNA sequences of oligonucleotides used in this study.

Primer[a]	Sequence
***eae*$_{EPEC}$ 5′ REGION**[b,c]	
F-pACYC184	5′ acc tga agt cag ccc cat acg ata 3′
R-*eae*$_{EPEC}$	5′ *agt aat cat* gtt tgg gct cca cca caa tg 3′
R-*eae*$_{EPEC}$-2	5′ *aac cat cat* gtt tgg gct cca cca caa tg 3′
***eae*$_{EPEC}$ 3′ REGION**[b,d]	
F-*eae*$_{EPEC}$	5′ *gtt tgt gta gaa taa* att taa taa ata tct aat cat tgt ccg gct aa 3′
R-pACYC184	5′ ccg ctc gag gct ctc cct tat gcg act cc 3′
F-*eae*$_{EPEC}$-2	5′ *gcg ctg tca ata taa* att taa taa ata tct aat cat tgt ccg gct aa 3′
R-pACYC184-2	5′ tga ctg ggt tga agg ctc tca agg 3′
OPEN READING FRAMES[b,e]	
F-*eae*$_{EHEC}$	5′ *agc cca aac* atg att act cat ggt tgt ta 3′
R-*eae*$_{EHEC}$	5′ *aga tat tta tta aat* tta ttc tac aca aac cgc at 3′
F-*inv*	5′ *agc cca aac* atg atg gtt ttc cag cca at 3′
R-*inv*	5′ *aga tat tta tta aat* tta tat tga cag cgc aca ga 3′
***C. RODENTIUM tir* MUTANT**[f,g]	
F-A-Tir-KO	5′ gtg cac aat cat caa tca gtc ac 3′
R-B-Tir-KO	5′ *acc aaa atc cct taa cgt gag tta cgc gtc gtt cca ctg agc gtc aga cc*a cat ata tcc ttt tat tta atc gga aat ttt aca cta atc c 3′
F-C-Tir-KO-zeo	5′ *ggt ctg acg ctc agt gga acg acg cgt aac tca cgt taa ggg att ttg gt* 3′
R-D-Tir-KO-zeo	5′ *tca gtc ctg ctc ctc ggc cac gaa gtg cac gca gtt gcc ggc cgg gtc gc* 3′
F-E-Tir-KO	5′ *gtg cac ttc gtg gcc gag gag cag gac tga* cgc tac aac acc ggg agt tga acg ttt cgt cta aat ata taa tgg gta ttt tgt tgg ggg gga ggg gga g 3′
R-F-Tir-KO	5′ ggc tcc acc aca atg agt tag 3′
F-Tir-Ext	5′ gct tgc atca taa gtt aga ctg tg 3′
R-Tir-Ext	5′ cgt cat tag tgt cag ata acg aga 3′
F-Tir-Int-323–342	5′ ttc gtg ttg aac agc agc ca 3′
R-Tir-Int-819–838	5′ tcg cag ctt ctg cag cat tt 3′
F-Tir-Int-590–610	5′ ata cac gtt ctg ttg gtg tcg 3′
R-Tir-Int-1049–1069	5′ cca att cct gct gac gtt tag 3′
F-Zeo-Int	5′ *gag cgg tcg agt tct gg* 3′
R-Zeo-Int	5′ *gcc acg aag tgc acg cag t* 3′

[a] *F, forward (top strand) primer; R, reverse (bottom strand) primer.*

[b] *Underlined sequences represent the restriction enzyme sites used for cloning. Italicized sequences indicate the tails used for fusing eae$_{EPEC}$ 5′ and 3′ sequences with eae$_{EHEC}$, inv, or hybrid allele coding regions.*

[c] *For pInt$_{EHEC}$, F-pACYC184, and R-eae$_{EPEC}$ were used. For all others, F-pACYC184 and R-eae$_{EPEC}$-2 were used.*

[d] *For pInv, F-eae$_{EPEC}$-2, and R-pACYC184-2 were used. For all others, F-eae$_{EPEC}$ and R-pACYC184 were used.*

[e] *For pInt$_{EHEC}$, F-eae$_{EHEC}$, and R-eae$_{EHEC}$ were used. For pInv, F-inv, and R-inv were used. For all others, F-inv and R-eae$_{EHEC}$ were used.*

[f] *Primers used to construct C. rodentiumΔtir. Italicized regions indicate homology to zeocin cassette.*

[g] *Numbers in primer name correspond to nucleotide positions primer is located within tir gene. Int represents screening primers internal to the gene (tir or zeocin) and Ext represents screening primers that are external to the gene.*

eae (∼2.8 kb) with 3′ EHEC tail, and EHEC 3′UTR (∼960 bp). The PCR product was cloned into pGEM7xf(+), linearized by *Bam*HI and *Xba*I digestion, and transformed into KM48 harboring pTP223. Transformants were screened by sucrose resistance and chloramphenicol sensitivity. Candidates were confirmed by PCR and sequencing.

GENERATION OF *C. RODENTIUM* Δ *TIR*

The *C. rodentium tir* deletion mutant was made using a slightly modified version of the one-step PCR-based gene activation protocol (Datsenko and Wanner, 2000). Briefly, a tertiary PCR product containing the zeocin cassette and its promoter flanked by 576 bp homology upstream of the start of *tir* and 608 base pairs downstream of the stop of *tir* was generated using three template PCR products, A + B, C + D, and E + F. Product A + B was generated using primers F-A-Tir-KO and R-B-Tir-KO (**Table 3**) with genomic *C. rodentium* (DBS100) DNA (isolated using a kit by Promega, Madison, WI, USA) as a template. Primers F-C-Tir-KO-zeo and R-D-Tir-KO-zeo were used to amplify a *Mlu*I/*Eco*RI cut pDONORzeo fragment to generate the 504-bp PCR product C + D. Primers F-E-Tir-KO and R-F-Tir-KO were used to amplify genomic *C. rodentium* DNA to make PCR product E + F. This 1.65 kb PCR product was electroporated into *C. rodentium* (DBS100) containing the lambda-red plasmid, pKD46, and recombinants were selected for by plating on LB plates supplemented with 750 μg/ml ampicillin. Replacement of *tir* with the zeocin

cassette and its promoter was confirmed by PCR using the following primers: F-Tir-Ext, R-Tir-Ext, F-Tir-Int-323–342, R-Tir-Int-819–838, F-Tir-Int-590–610, R-Tir-Int-1049–1069, F-Zeo-Int, and R-Zeo-Int (see **Table 3**).

PURIFICATION OF EHEC TIR AND GENERATION OF AN ANTI-EHEC TIR ANTIBODY

pDV205 (amp^R) is a derivative of pET21 that contains EHEC *tir* with a histidine tag. For expression and purification of EHEC Tir, BL21 DE3+ pDV205 was cultured in 2x YT media at 37˚C to an OD_{600} of 0.6–0.7 then induced with 1 mM IPTG for 3 h at 37˚C. The culture was spun at 4,420 × g, 20 min at 4˚C, and the supernatant was discarded. The pellet was resuspended in 2.5 ml lysis buffer per gram wet weight. Four milligrams of lysozyme was added and the sample was sonicated (Branson Sonifier 450, Branson Ultrasonics Corporation, Danbury, CT, USA) Duty cycle 70, output 3, 10 s bursts × 6 cycles). The lysate was then centrifuged at 16,100 × *g*, 20 min at 4˚C, and the supernatant was run on a QIAgen Ni-NTA Agarose column (QIAgen, Valencia, CA, USA) and the resulting EHEC Tir was eluted and quantified using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

For antibody production, all inoculations were completed subcutaneously with 30 ng of purified EHEC Tir in 50 μl with 50 μl of adjuvant for a total inoculation volume of 100 μl. Female BALB/c mice (Jackson Laboratories, Bar Harbor, ME, USA) were inoculated with EHEC Tir supplemented with Imject Freund's Complete Adjuvant (Thermo Scientific, Rockford, IL, USA). At days 14 and 28, mice were boosted with EHEC Tir supplemented with Imject Freund's Incomplete Adjuvant (Thermo Scientific, Rockford, IL, USA). Mice were sacrificed on day 38 and blood was harvested via cardiac bleed, allowed to clot at room temperature for 30 min, and then was spun twice at 8,600 × *g*, 15 min at 4˚C. Serum was then aliquoted and stored at −80˚C.

INVASION ASSAYS

Entry into cultured cells was measured in a manner similar to that described previously (Finlay and Falkow, 1988). Briefly, HEp-2 cells (ATCC CCL-23) were seeded in 24-well plates and grown overnight at 37˚C in 5% CO_2 and Dulbecco's Modified Eagles medium supplemented with 10% heat inactivated calf serum and 2 mM L-glutamine (DMEM). Bacteria were grown overnight in LB, diluted in fresh cell culture medium, and inoculated at an approximate multiplicity of infection (MOI) of 100. The cells were incubated for 3 h at 37˚C in 5% CO_2 and then washed with phosphate-buffered saline (PBS). Fresh cell culture medium supplemented with gentamicin was added to each well and the cells were incubated at 37˚C in 5% CO_2 for 1 h. The cells were washed with PBS, lysed with 1% Triton X-100 in PBS, and diluted to 1 ml with LB broth. Percent invasion for each well was determined by plating dilution series.

INFECTION OF MONOLAYERS PRE-INFECTED WITH EPEC *EAE*

The "prime and challenge" assay is a modification of a previously described bacterial adherence assay (Rosenshine et al., 1996; Liu et al., 1999) and was used to quantify intimin–Tir interactions *in vitro*. Briefly, nearly confluent monolayers of HEp-2 cells were washed twice in PBS, and RHFM [RPMI-1640 supplemented with 20 mM HEPES, pH 7.0, 2% fetal bovine serum (FBS), and 0.5% D-mannose] was added to the monolayers. The monolayers were then mock-infected or infected with the EPEC *eae* mutant strain JPN15.96 at MOI of ∼200 and incubated at 37˚C in 5% CO2 for 3 h. JPN15.96 is capable of high efficiency translocation of Tir into mammalian cells, but unable to form pedestals due to the absence of intimin. The monolayers were washed twice with PBS and then incubated for 1 h at 37˚C in 5% CO_2 with 100 μg/ml gentamicin in DMEM/HEPES to kill remaining bacteria. The monolayers were washed three times in PBS and then infected with the appropriate "challenge" bacterium (i.e., *C. rodentium*, *C. rodentium*Δ*eae*, or *C. rodentium*Δ*eae* expressing EPEC intimin, EHEC intimin, invasin, or an invasin–intimin hybrid) diluted in fresh DMEM/HEPES at an MOI of 100 for 3 h at 37˚ in 5% CO_2. At the conclusion of the assay, monolayers were washed six times with PBS, lysed with 1% Triton X-100 in PBS, and diluted to 1 ml with LB broth. Percent adherence for each well was determined by plating dilution series.

The prime and challenge assay was also adapted to evaluate actin pedestal formation mediated by intimin and its derivatives by seeding HEp-2 cells onto coverslips. At the conclusion of the assay described above, coverslips were washed six times with PBS, fixed with 2.5% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Coverslips were then double fluorescently labeled for F-actin and bacteria and examined on a Nikon Eclipse E600 microscope. F-actin was labeled with Texas red-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) and visualized with a 580-nm dichroic filter. *C. rodentium* were labeled with an anti-*C. rodentium* polyclonal rabbit antibody raised against *C. rodentium* strain DBS100 and visualized with a Cascade blue-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA) using a 400-nm dichroic filter. Coverslips labeled only with anti-*C. rodentium* primary antibody and the Cascade blue-conjugated secondary antibody showed no crossover when viewed with the 580-nm filter. Image acquisition was performed with the Spot program (Diagnostics Instruments, software version 3.0.4) and imported into Adobe Photoshop 5.0.

PEDESTAL, BACTERIAL BINDING, AND TIR FOCUSING ASSAYS

The FAS assay of (Nicholls et al., 2000), as modified for *C. rodentium* (Newman et al., 1999) was the basis for this assay. A single colony from each strain was grown in 1 ml media (+/− antibiotic) for 8 h. Cultures were diluted 1:500 into 5 ml DMEM supplemented with 0.1 M HEPES (pH 7.0; +/− antibiotic) and incubated at 37˚C without agitation with 5% CO_2 for 12–15 h. Cell monolayers were prepared by splitting 95–100% confluent mouse embryonic fibroblasts (MEFs) into 24-well culture plates containing sterile glass coverslips followed by overnight growth at 37˚C with 5% CO_2. Prior to seeing onto culture plates, MEFs were maintained in MEF cell culture media [DMEM (hi glucose) + 10% FBS with penicillin, streptomycin, and glutamine] at 37˚C, 5% CO_2. For infections, cell monolayers were washed twice with sterile PBS followed by addition of FAS media containing 25 μl of overnight cultured *C. rodentium* to each well. Plates were spun at 700 g for 10 min then incubated at 37˚C with 5% CO_2 for 3 h. After 1.5 h, plates were spun again at 700 g for an additional 10 min to insure proper bacterial binding to cells. After 3 h, cells were washed twice with sterile PBS and 0.5 ml pre-warmed FAS media

was added to each well and cells were then incubated for an additional 3 h. Cells were then washed five times with sterile PBS, fixed with 4% PFA for 30 min, washed, permeabilized with 0.1% Triton X-100, and stained with anti-EHEC Tir antibody (1:500) as a primary antibody for 30 min. The samples were washed and then stained for 30 min with anti-Mouse 488 secondary antibody (Invitrogen, Carlsbad, CA, USA; 1:200), phalloidin (Molecular Probes, OR, USA; 1:100), and DAPI (1:500). After washing cells an additional three times with PBS, the coverslips were mounted on slides using ProLong® Gold anti-fade reagent (Invitrogen, Eugene, OR, USA). For binding assays, the number of bacteria per cell was counted for 25 random cells. Counting was done in triplicate and the mean, median, and maximum number of bacteria bound to cells was determined. The stratification of the number of cells with the given amount of bound bacteria in each interval (0, 1–5, 6–10, 11–15, 16–20, and >20) was also determined.

IMMUNOBLOTTING

Preparation of bacterial cell lysates was performed as described previously (Campellone et al., 2002; Brady et al., 2007). Samples were resolved by 10% SDS PAGE and transferred to PVDF membrane. Membranes were blocked in PBS supplemented with 5% milk for 30 min prior to treatment with sheep anti-EHEC Intimin (1:500), goat anti-EPEC Intimin (1:2000), rabbit anti-OmpA, or rabbit anti-O157 antiserum (1:750, Difco Laboratories, Lawrence, KS, USA) for 2 h, washed and treated with secondary antibody as previously described (Campellone et al., 2004a; Campellone and Leong, 2005; Brady et al., 2007).

IMMUNOFLUORESCENCE MICROSCOPY OF EHEC STRAINS

To ensure an EHEC strain expressing EPEC intimin can generate actin pedestals and that intimin plasmids used to overexpress EHEC and EPEC intimin are functional to complement a deletion of EHEC intimin by FAS, HeLa cell monolayers were infected with indicated strains, fixed, and permeabilized as described previously (Campellone et al., 2002). For each strain, qualitative scoring of F-actin pedestals was performed as indicated. Approximately equal numbers of actin pedestals were observed for EHECΔ*eae* expressing EHEC intimin (pHL6) or EPEC intimin (pHL4).

MOUSE INFECTION STUDIES

Mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in the UMMS animal facility. All animal procedures were done in compliance with the University of Massachusetts Medical School IACUC. Female, eight-week-old C57BL/6J mice were gavaged with PBS or $\sim 2 \times 10^9$ of overnight culture of *C. rodentium* strain specified in 100 µl PBS. Inoculum concentrations were confirmed by serial dilution plating. *C. rodentium* fecal shedding was determined by serial dilution plating of fecal slurries (10% w/v in PBS) on Maconkey agar or LB agar with selection for zeocin, kanamycin, chloramphenicol, or kanamycin and chloramphenicol. For streptomycin pre-treatment experiments, mice were given 5 mg/ml streptomycin for 48 h prior to infection.

MOUSE TISSUE COLLECTION AND HISTOLOGY

At necropsy the large intestine (colon through cecum) was fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Five-micron sections stained with hematoxylin and eosin were evaluated and scored blindly by a board-certified pathologist (Vijay Vanguri). Assessment of mucosal hyperplasia was targeted to areas of comparable muscularis propria thickness in order to reduce error from differences in planes of section.

TRANSMISSION ELECTRON MICROSCOPY

Mouse intestinal tissue samples were taken at various time points post-infection and fixed in 2.5% glutaraldehyde in 0.05 M Sodium Phosphate buffer, pH 7.2. Samples were processed and analyzed at the University of Massachusetts Medical School Electron Microscopy core facility according to standard procedures. Briefly, fixed samples were moved into fresh 2.5% glutaraldehyde in 0.05 M Sodium Phosphate buffer and left overnight at 4°C. The samples were then rinsed twice in the same fixation buffer and post-fixed with 1% osmium tetroxide for 1 h at room temperature. Samples were then washed twice with DH_2O for 20 min at 4°C and then dehydrated through a graded ethanol series of 20% increments, before two changes in 100% ethanol. Samples were then infiltrated first with two changes of 100% Propylene Oxide and then with a 1:1 mix of propylene oxide: SPI-Pon 812 resin. The following day three changes of fresh 100% SPI-Pon 812 resin were done before the samples were polymerized at 68°C in plastic capsules. The samples were then reoriented and thin sections were placed on copper support grids and stained with lead citrate and uranyl acetate. Sections were examined using the FEI Tecani 12 BT with 80 kV accelerating voltage, and images were captured using a Gatan TEM CCD camera.

PIGLET INFECTIONS

Gnotobiotic piglets were derived and infected orally with TUV93-0, which is deficient for the expression of Stx, thus eliminating the potentially confounding toxigenic effects on colonization (Tzipori et al., 1995), as described previously (Campellone et al., 2007; Ritchie et al., 2008; Brady et al., 2011). To quantify bacteria in the small intestine, the small intestine was divided into five parts and each section was viewed individually for the presence of bacteria and AE lesions. If bacteria or AE lesions were found in any of these sections, they were given the score of "+." The average score was then determined for the small intestines of piglets in each group. A score of "+" indicates the presence of bacteria and AE lesions in at least one of the five sections of the small intestine in each of the piglets, a score of "+/−" indicates the presence of bacteria and AE lesions in at least one of the five sections of the small intestine in at least half of the piglets, and a score of "−" indicates that fewer than half of the piglets had bacteria and AE lesions in at least one of the five sections of the small intestine and also includes piglets that did not become colonized. To enumerate bacteria in the spiral colon and cecum, each section was scored for the presence of bacteria and AE lesions. A score of "+" indicates that every piglet in the group had bacteria and AE lesions present, a score of "+/−" indicates that at least half of the piglets in the group had bacteria and AE lesions present, and a score of "−" indicates that fewer than half of the piglets had bacteria and AE lesions present.

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism. Comparison of multiple groups was performed using one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-tests.

Statistical significance of differences between two groups was evaluated using two-tailed unpaired *t*-tests. In all tests *p*-values below 0.05 (*), 0.01 (**), and 0.001 (***) were considered statistically significant, unless indicated otherwise. In all graphs, error bars represent SEM.

RESULTS

PRECISE CHROMOSOMAL REPLACEMENT OF EHEC *EAE* WITH EPEC *EAE* DOES NOT ALTER TISSUE TROPISM IN PIGLETS

Plasmid complementation of an EHEC *eae* mutant with EPEC *eae* results in a strain with altered tissue tropism that colonizes the small bowel of piglets (Tzipori et al., 1995). To determine if EPEC and EHEC intimin might be functionally interchangeable when expressed from the endogenous chromosomal locus, we precisely replaced the EHEC *eae* allele in EHEC TUV93-0 with the *eae* allele from EPEC strain JPN15/pMAR7 (**Figure 1A**). Immunoblotting confirmed that the resulting strain, LM-1, produced EPEC intimin (**Figure 1B**). As predicted from previous work demonstrating that EHEC and EPEC intimin are functionally interchangeable for *in vitro* pedestal formation (DeVinney et al., 1999), LM-1 generated actin pedestals on cultured cells (**Figure 1C**).

To test the function of EPEC intimin during infection by EHEC, gnotobiotic piglets were infected orally with LM-1 and after 72 h, infection was assessed by histological analysis of the small and large intestines. Upon infection with wild type EHEC, piglets suffered diarrheal illness and bacteria were associated with extensive segments of the cecal and colonic epithelium (**Table 4**). Wild type EHEC were only occasionally associated with the epithelium of the small intestine. In contrast, infection of gnotobiotic piglets with EHEC Δ*eae* did not result in detectable bacterial association with epithelium of any intestinal segment, and correspondingly, the animals did not suffer from diarrhea. Notably, EHEC strain LM-1, which expresses EPEC intimin, colonized the epithelium of the cecum and spiral colon at levels indistinguishable from that of wild type EHEC, and like the wild type strain, induced diarrheal illness (**Table 4**). In contrast to the previous finding that EHEC expressing EPEC intimin from a plasmid could efficiently colonize the small intestine of gnotobiotic piglets (Tzipori et al., 1995), bacteria were vanishingly sparse in the small intestine (**Table 4**). These results demonstrate that EPEC intimin, when expressed in EHEC from the endogenous chromosomal locus, can provide intimin function during intestinal infection of piglets, but does not influence tissue tropism in this model.

EHEC INTIMIN CAN PROMOTE MURINE COLONIZATION AND DISEASE BY *C. RODENTIUM*

We further assessed ability of EHEC intimin to complement a *C. rodentium*Δ*eae* mutant for colonization and intestinal disease in mice. Previous work demonstrated that *C. rodentium*Δ*eae* harboring pCVD438, which encodes EPEC intimin, was fully virulent in Swiss NIH and C3H/HeJ mice (Hartland et al., 2000; Mundy

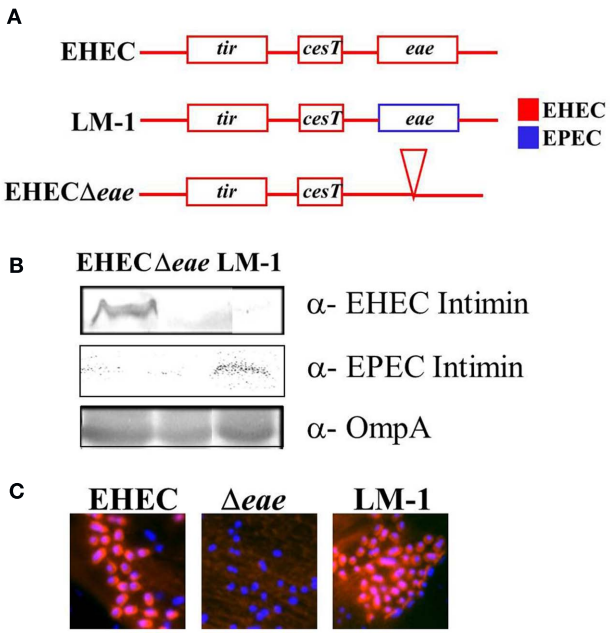

FIGURE 1 | An EHEC strain containing EPEC intimin on the chromosome is functional for pedestal formation. (A) The endogenous EHEC *eae* gene was precisely replaced by EPEC *eae* to create LM-1. **(B)** Expression of EHEC and EPEC intimin in wild type EHEC, EHEC Δ*eae,* and LM-1 was assessed by western blot. Blotting with anti-OmpA was included as a loading control. **(C)** HeLa cells were infected with the indicated strain and the monolayers were stained with DAPI (blue) to visualize bound bacteria and phalloidin (red) to visualize actin pedestals.

Table 4 | An EHEC strain carrying a precise chromosomal replacement of *eae* with EPEC *eae* displays piglet intestinal tropism indistinguishable from wild type.

***E. coli* strain**	**No. of animals**	**No. of animals with diarrhea**	**AE lesions and colonization**		
			Small intestine	**Cecum**	**Spiral colon**
WT EHEC	5	5	+/–	+	+
EHEC Δ*eae*	4	0	–	–	–
EHEC LM-1	4	4	+/–	+	+

*Gnotobiotic piglets were infected orally with 5 × 10⁹ EHEC, EHEC*Δeae*, or LM-1. Animals were sacrificed 72 h after infection and the intestines were removed, fixed, stained, and scored for colonization using a score adapted from Tzipori et al., 1995; see Materials and Methods. "+" indicates bacteria and AE lesions present, "+/–" indicates minimal bacteria and AE lesions present, and "–" indicates no bacteria or AE lesions present. Values represent the average colonization score of all the piglets in the group.*

et al., 2007). In contrast, *C. rodentium*Δ*eae* expressing an intimin hybrid carrying the N-terminal 554 residues of EPEC intimin and the C-terminal 380 residues of EHEC intimin colonized mice 100-fold less efficiently and did not cause the colonic hyperplasia that is characteristic of *C. rodentium* infection (Hartland et al., 2000; Mundy et al., 2007).

To compare the colonization function of EPEC and EHEC intimin in mice, we precisely replaced the EPEC *eae* coding sequence of pCVD438 (which for simplicity we herein refer to as "pInt$_{EPEC}$") with the EHEC *eae* coding sequence to create pInt$_{EHEC}$ (i.e., pHL69; **Table 2**). C57BL/6 mice were infected with approximately 5×10^9 CFU of *C. rodentium*, *C. rodentium*Δ*eae*, *C. rodentium*Δ*eae*/pInt$_{EPEC}$, or *C. rodentium*Δ*eae*/pInt$_{EHEC}$. Mice infected with *C. rodentium* reached peak colonization levels seven days post-infection with approximately 10^9 bacteria per gram of feces before being cleared from mice by approximately 15–20 days after inoculation, presumably due to the development of an adaptive immune response (Ghaem-Maghami et al., 2001; Vallance et al., 2002; Simmons et al., 2003; **Figure 2A** and data not shown). In contrast, the number of *C. rodentium*Δ*eae* in the stool peaked at three days post-infection, reaching less than 10^6 bacteria per gram of feces, and quickly diminished, never rising to more than 10^4 bacteria per gram of feces thereafter (**Figure 2A**). Similar to what was previously reported (Frankel et al., 1996b; Hartland et al., 2000; Mundy et al., 2007), EPEC intimin, when expressed in *C. rodentium*Δ*eae* promoted high level colonization with comparable kinetics to wild type *C. rodentium* (**Figure 2A**). Additionally, intestinal sections from rodents infected with *C. rodentium*Δ*eae* expressing EPEC intimin showed colonic mucosal hyperplasia, goblet cell depletion, acute inflammation, erosions, and degenerative epithelial changes on the surface and in the crypts (**Figure 2B**). Interestingly, we also found that EHEC intimin, when expressed in *C. rodentium*Δ*eae*, promoted colonization, colonic hyperplasia, and intestinal damage indistinguishable from wild type *C. rodentium* (**Figure 2**) and generated AE lesions morphologically identical to wild type *C. rodentium* (**Figure 3**). Thus, EHEC intimin, in spite of being significantly divergent in sequence from *C. rodentium* intimin, could provide complete intimin function in this model.

EHEC INTIMIN AND TIR-BINDING INVASIN–INTIMIN FUSIONS CAN MEDIATE HOST CELL ADHERENCE BY *C. RODENTIUM*

We next characterized the ability of *C. rodentium*Δ*eae* expressing EHEC intimin to attach to host cells that express high levels of Tir on their surface, which more sensitively assesses Tir–intimin interactions compared to conventional infection assays (Liu et al., 2002). Pre-infection (i.e., "priming") of cells with an EPECΔ*eae* mutant permits efficient delivery of Tir to the eukaryotic cell (Rosenshine et al., 1996; Liu et al., 1999). After gentamycin treatment and washing to kill and remove bound bacteria, infection (i.e., "challenge") of these monolayers with intimin-expressing bacteria permits sensitive assessment of bacterial binding mediated by Tir–intimin interaction (Rosenshine et al., 1996; Liu et al., 1999). Utilizing this assay, *C. rodentium*Δ*eae*/pInt$_{EHEC}$ bound to pre-infected monolayers indistinguishably from that of wild type *C. rodentium*, and approximately 20-fold more efficiently than *C. rodentium*Δ*eae* (**Figure 4A**).

We next characterized the minimal region of EHEC intimin required for Tir-mediated cell adherence when expressed in *C. rodentium*. The intimin related protein *Y. pseudotuberculosis* invasin, which binds to β_1-chain integrins (Isberg and Leong, 1990; Frankel et al., 1996a), was previously used to deliver various

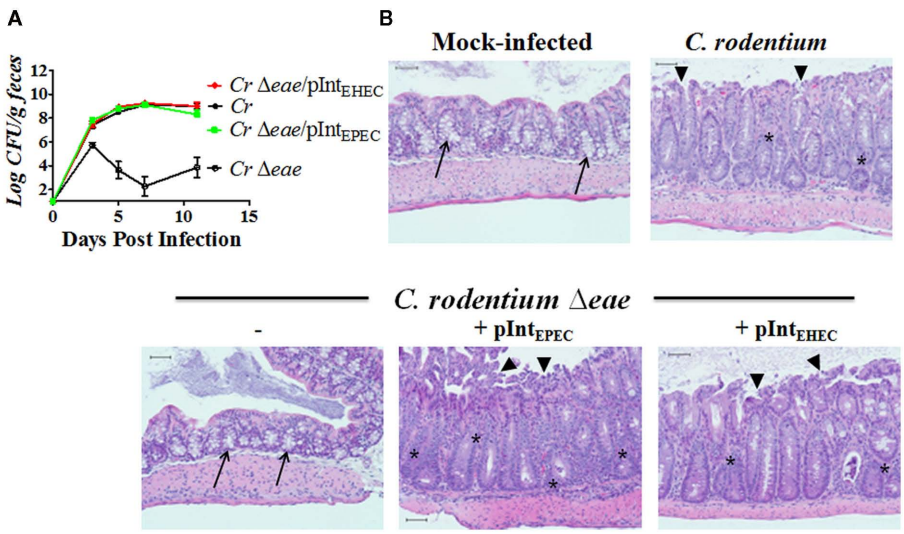

FIGURE 2 | Enterohemorrhagic *E. coli* intimin is able to restore colonization and disease in a *C. rodentium eae* mutant. **(A)** Colonization of eight-week-old C57BL/6 mice by *C. rodentium* ("*Cr*"), *C. rodentium*Δ*eae*, *C. rodentium*Δ*eae*/pInt$_{EPEC}$, or *C. rodentium*Δ*eae*/pInt$_{EHEC}$ was determined by viable stool counts. Shown are the averages CFU (±SEM) of five mice. Data shown are representative of one of three independent experiments. **(B)** Hematoxylin and eosin stained large intestinal sections from mock-infected mice or mice infected with indicated strain are shown under 100× magnification. Arrow heads point to ragged areas of the surface epithelium at sites of surface erosion. Arrows point to areas of many goblet cells. Asterisks indicate areas of active inflammation. Scale bars measure 50 μm.

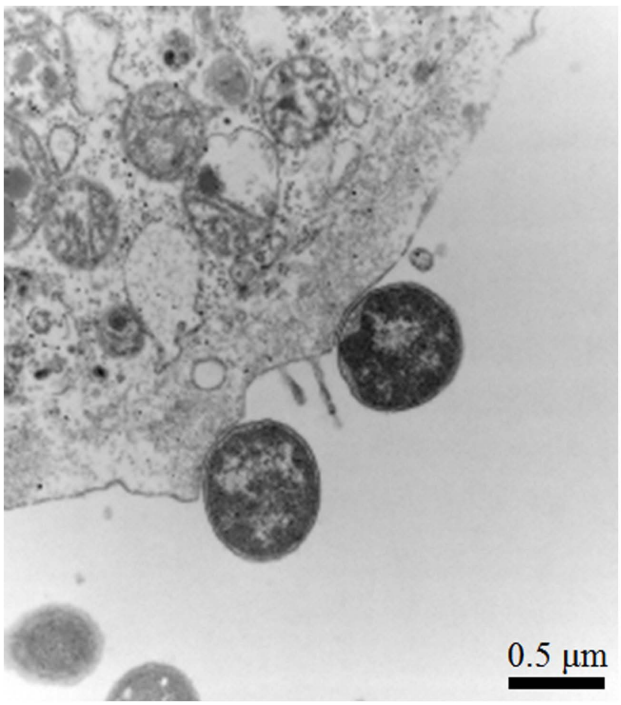

FIGURE 3 | Enterohemorrhagic *E. coli* intimin can complement a *C. rodentium eae* mutant for AE lesion formation *in vivo*. Transmission electron micrograph showing AE pedestal formation on colonic epithelial cells from the distal colon of mice infected with *C. rodentium*Δ*eae*/pInt$_{EHEC}$. Original magnification, 25,000×. Bar, 0.5 μm.

portions of EHEC intimin to the surface of the bacteria in the form of invasin–intimin hybrid proteins (Liu et al., 1999). *C. rodentium*Δ*eae* expressing invasin was capable of high efficiency entry into cultured epithelial cells, strongly suggesting that invasin can be expressed in functional form on the surface of *C. rodentium* (data not shown). Thus, we determined the minimal region of EHEC intimin required for cell adherence by *C. rodentium* by expression of invasin–intimin fusions in this bacterium. *C. rodentium*Δ*eae* producing Inv–Int395, a hybrid that contains the C-terminal 395 amino acids of intimin, bound to pre-infected monolayers at wild type levels, implying that this hybrid promotes Tir-binding when expressed in *C. rodentium*Δ*eae*, as it does when expressed in *E. coli* K12 (**Figure 4A**; Liu et al., 1999). In contrast, Inv–Int181, which was previously shown to retain some Tir-binding activity when expressed in *E. coli* K12 (Liu et al., 1999), and Inv–Int100, bound poorly to monolayers pre-infected with the EPECΔ*eae* mutant (**Figure 4A**). These data suggest that the minimal functional domain of EHEC intimin that can confer high level Tir-binding on *C. rodentium* is located in the C-terminal 395 amino acids.

To determine if the functional Tir-binding domain defined by the more elaborate prime and challenge assay described above also functioned to promote cell attachment by *C. rodentium* during simple infection, we infected MEFs with *C. rodentium*Δ*eae* expressing invasin or EHEC intimin derivatives and determined the number of bacteria bound to each of 25 randomly selected cells. Cells infected with wild type *C. rodentium* harbored an average of more than nine bacteria per cell, and fewer than five of the 25 cells were entirely free of bacteria (**Figure 4B**). Intimin was required for binding, because approximately 20 of the 25 cells infected with *C. rodentium*Δ*eae* were bacteria-free, and virtually no cells harbored more than five bacteria (**Figure 4B**). EHEC intimin, when expressed in *C. rodentium*Δ*eae*, was able to restore binding capabilities of the mutant strain, with an average of approximately six bacteria per cell (**Figure 4B**).

We similarly tested the ability of the invasin–intimin hybrids to bind to cells. Invasin, when expressed on the surface of *C. rodentium*Δ*eae*, dramatically enhanced the ability of the bacteria to associate with cells ($p < 0.05$), with an average of approximately 19 bacteria per cell, consistent with its ability to promote high-level host cell interaction (**Figure 4B**; Isberg and Leong, 1990). *C. rodentium*Δ*eae* expressing Inv–Int395, which mediated attachment of *C. rodentium* in the prime and challenge assay (**Figure 4A**), bound to mammalian cells statistically indistinguishably from wild type *C. rodentium*, with an average of approximately eight bacteria bound to each cell (**Figure 4B**). On average, fewer than five of the 25 cells were bacteria-free. In contrast, *C. rodentium* expressing Inv–Int181 and Inv–Int100 bound to cells indistinguishably from *C. rodentium*Δ*eae*, with an average of less than two bacteria per cell and the majority of cells harboring fewer than five bacteria (**Figure 4B**). These data indicate that EHEC intimin can complement a *C. rodentium*Δ*eae* mutant for cell attachment, and that the binding function of this protein is encompassed by the C-terminal 395 amino acids.

EHEC INTIMIN, BUT NOT A TIR-BINDING INVASIN–INTIMIN HYBRID, PROMOTES TIR CLUSTERING AND PEDESTAL FORMATION BY *C. RODENTIUM*

Given the ability of EHEC intimin and the hybrid Inv–Int395 to promote binding of *C. rodentium* to mammalian cells, we next tested their ability to promote Tir clustering and pedestal formation upon infection of mammalian cells. As expected, cells infected with *C. rodentium*Δ*eae* demonstrated virtually no bound bacteria and after staining monolayers with anti-EHEC Tir, only one quarter of the rare bound bacteria were associated with somewhat diffuse foci of Tir (**Figure 5**, column 2; and data not shown). In contrast, upon infection with wild type *C. rodentium,* 95% of bound bacteria were associated with intensely stained Tir foci, and 98% of bacteria generated actin pedestals (**Figure 5**, "*Cr*"). These defects in Tir clustering and pedestal formation were fully complemented by a plasmid expressing EHEC intimin, as well as by a plasmid expressing EPEC intimin, which was included as a positive control (**Figure 5**, "pInt$_{EHEC}$" and "pInt$_{EPEC}$", respectively). Thus, EHEC intimin appears to provide full function for cell binding, Tir clustering, and pedestal formation when expressed in *C. rodentium*.

To define the region of EHEC intimin critical for Tir clustering and pedestal formation, in parallel we infected monolayers with *C. rodentium*Δ*eae* expressing invasin–intimin hybrids. *C. rodentium*Δ*eae* expressing invasin or either of the two fusion proteins, Inv–Int181 or Inv–Int100, that were unable to promote Tir-mediated cellular attachment by *C. rodentium*, resulted in detectable Tir foci no more frequently than was found for *C. rodentium*Δ*eae* (**Figure 5**, "pInv", "pInv–Int181", or "pInv–Int100"). In contrast, 59% of *C. rodentium*Δ*eae* expressing Inv–Int395, which

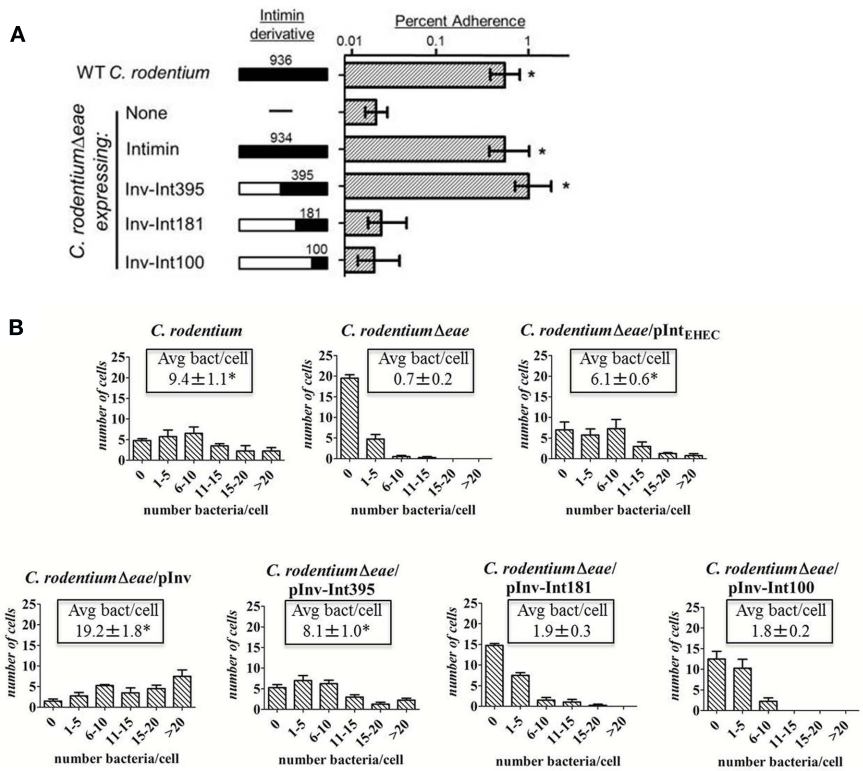

FIGURE 4 | A hybrid containing the C-terminal 395 amino acids of EHEC intimin can complement a *C. rodentium eae* mutant for host cell attachment. (A) HEp-2 cells pre-infected with an EPEC Δ*eae* strain were infected with wild type *C. rodentium*, *C. rodentium*Δ*eae*, or *C. rodentium*Δ*eae* strains expressing EHEC intimin, invasin, or the indicated invasin–EHEC intimin hybrids (Inv–Int395, Inv–Int181, and Inv–Int100), and stably bound bacteria were determined (Materials and Methods). Data are shown as the mean ± SEM and represent two independent assays with at least three replicates per assay. Asterisk indicates binding significantly ($p < 0.05$) higher than *C. rodentium*Δ*eae* of *C. rodentium*Δ*eae* expressing pInv–Int181 or pInv–Int100, determined by two-tailed unpaired *t*-test. **(B)** Mouse embryonic fibroblasts were infected with the indicated *C. rodentium* strains and the number of bound bacteria per cell was counted for four sets of 25 randomly selected cells. Shown are the number of cells with the given number (0, 1–5, 6–10, 11–15, 16–20, and >20) of bound bacteria. The experiment was performed in triplicate. Shown in box is the mean number of bacteria per cell ± SEM. Asterisk indicates a significant difference ($p < 0.05$) compared to *C. rodentium*Δ*eae*, determined using a one-way ANOVA test.

promotes binding to *C. rodentium* to mammalian cells (**Figure 4**), were associated with Tir foci (**Figure 5**, "pInv–Int395"), a percentage that was both significantly higher than that of *C. rodentium*Δ*eae* and significantly lower than that of wild type *C. rodentium* or *C. rodentium*Δ*eae* expressing wild type EHEC intimin. The partial complementation of Tir clustering by pInv–Int395 was also reflected in the low (i.e., 7%) frequency of pedestal formation (**Figure 5**, "pInv–Int395"). Thus, although the expression of Inv–Int395 in *C. rodentium* resulted in high-level Tir-mediated cell binding, it did not result in efficient Tir clustering or pedestal formation in cultured mammalian cells.

A TIR-BINDING INVASIN–INTIMIN FUSION PROTEIN DOES NOT PROMOTE MURINE COLONIZATION *BY C. RODENTIUM* AND RESULTS IN MINIMAL COLONIC HYPERPLASIA

After characterizing the invasin–intimin hybrids *in vitro*, we next determined their ability to complement a *C. rodentium*Δ*eae* mutant for colonization and disease *in vivo*. As shown above (**Figure 2A**), after oral gavage of C57BL/6 mice with approximately 5×10^9 C. *rodentium*Δ*eae*/pInt$_{EHEC}$, *C. rodentium*Δ*eae* expressing EHEC intimin demonstrated wild type levels of colonization, colonic hyperplasia, goblet cell depletion, and abundant inflammation (**Figures 6A,B**). In contrast, in most instances *C. rodentium*Δ*eae*/pInv displayed colonization kinetics indistinguishable from *C. rodentium*Δ*eae* (**Figure 6A**) and did not trigger colitis (**Figure 6B**), showing that bacterial binding to β_1-chain integrins, even by a high affinity ligand such as invasin (Tran Van Nhieu and Isberg, 1993) is not sufficient to promote *C. rodentium* colonization. (Occasionally, *C. rodentium*Δ*eae*/pInv colonized mice somewhat better than *C. rodentium*Δ*eae*, but always at a level several orders of magnitude lower that wild type *C. rodentium* or *C. rodentium* expressing EHEC intimin and usually just above the limit of detection, i.e., 100 CFU/g feces; data not shown).

As expected, *C. rodentium*Δ*eae* expressing Inv–Int181 or Inv–Int100, neither of which mediated mammalian cell attachment, Tir clustering, or pedestal formation *in vitro*, colonized mice no better than *C. rodentium*Δ*eae* and resulted in no detectable intestinal histopathology (**Figures 6A,B**). Notably, *C. rodentium*Δ*eae* expressing Inv–Int395, which promoted Tir-mediated attachment to cultured monolayers but did not cluster Tir efficiently or trigger pedestal formation on cultured cells, was as defective as *C. rodentium*Δ*eae* in colonization and triggered

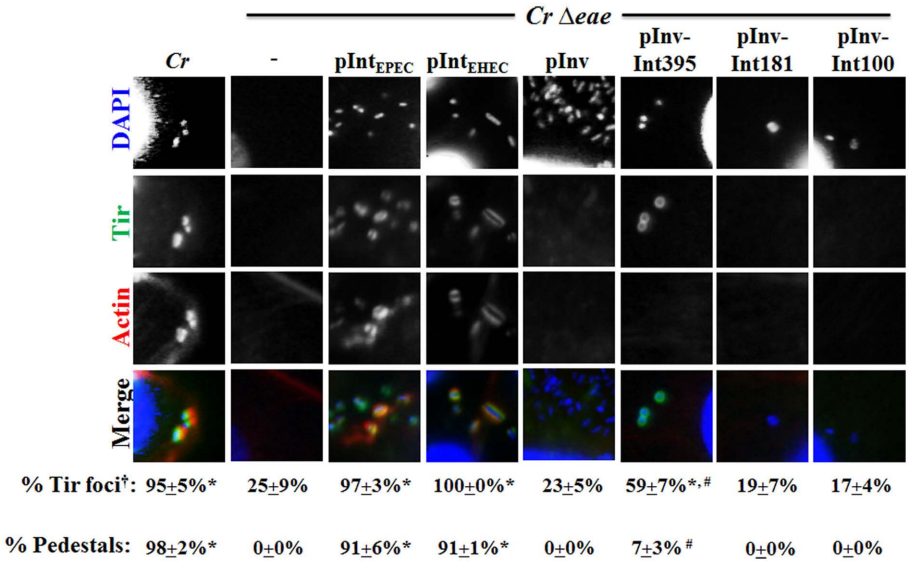

FIGURE 5 | Enterohemorrhagic *E. coli* intimin, but not a Tir-binding invasin–intimin hybrid, promotes Tir clustering and pedestal formation by *C. rodentium*. Monolayers were infected with the indicated *C. rodentium* ("*Cr*") strains and stained with DAPI to visualize bacteria (blue), anti-EHEC Tir antibody (green), and fluorescent phalloidin (red) to detect F-actin. Twenty-five bound bacteria were scored for their association with Tir foci or pedestals. †Note that when quantifying the percentage of cells infected with *C. rodentium*Δ*eae* that had Tir foci and/or actin pedestals associated with bound bacteria, many more cells needed to be counted given the inability of this strain to adhere efficiently to cells. Data represent quadruplicate samples. Asterisk indicates that percent positive bacteria is significantly ($p < 0.05$) greater than that for *C. rodentium*Δ*eae*. "#" Indicates that percent positive bacteria is significantly ($p < 0.05$) lower than that for *C. rodentium*/pInt$_{EHEC}$.

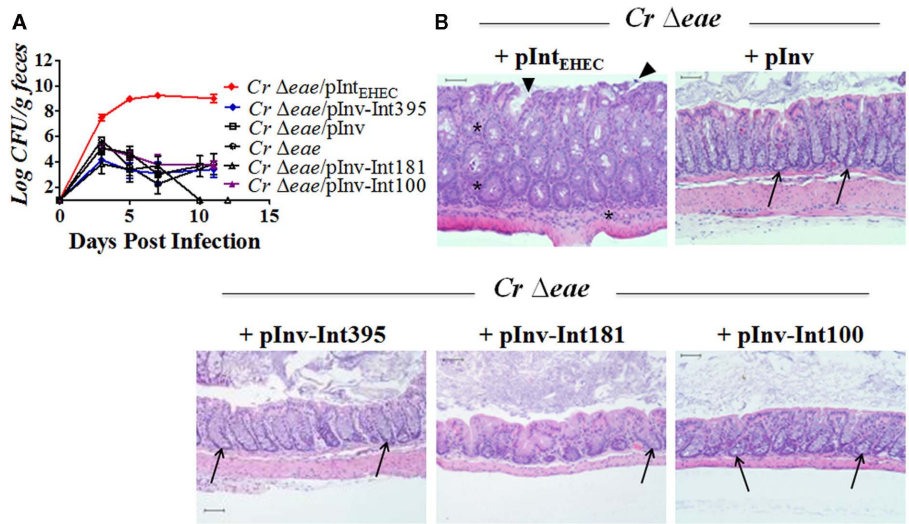

FIGURE 6 | A Tir-binding invasin–intimin fusion protein does not promote murine colonization by *C. rodentium*. (A) Eight-week-old C57BL/6 mice were inoculated with indicated *C. rodentium* strains by oral gavage and intestinal colonization was determined by viable stool counts. Shown are the averages CFU (±SEM) of groups of five mice. Each strain was tested in at least three independent experiments. The data displayed includes data from two different experiments, one in which *C. rodentium*Δ*eae*/pInv and *C. rodentium*Δ*eae* were analyzed and another in which the remaining strains were tested. The combined results are overlaid on the graph for ease of comparison. **(B)** Hematoxylin and eosin stained large intestinal sections from mice infected with designated strain were analyzed upon 100× magnification. Scale bars measure 50 μm. Arrow heads indicate foci of epithelial surface disruption. Arrows indicate areas of goblet cells and asterisks indicate foci of active inflammation.

virtually no intestinal damage (**Figures 6A,B**). The inability of Inv–Int395 to demonstrate wild type intimin function during mammalian infection demonstrates that the ability of intimin to bind Tir, in the absence of efficiently clustering Tir and triggering pedestal formation, is insufficient to promote colonization and disease.

INTIMIN BUT NOT TIR IS REQUIRED FOR *C. RODENTIUM* COLONIZATION OF MICE PRE-TREATED WITH STREPTOMYCIN

Streptomycin pre-treatment of mice permits EHEC, which is not normally an efficient mouse pathogen, to both colonize mice and cause toxigenic disease (Wadolkowski et al., 1990a,b; Melton-Celsa et al., 1996; Mohawk and O'Brien, 2011). Pilot experiments indicated that brief streptomycin pre-treatment of out-bred Swiss-Webster mice facilitated *C. rodentium* infection (David B. Schauer, Joseph Newman, and Steven A. Luperchio, unpublished observations), so we pre-treated C57BL/6 mice with streptomycin for 48 h prior to infection with *C. rodentium* strains that display different capacities to generate actin pedestals. Wild type *C. rodentium* colonized these mice with high efficiency, reaching $\sim 10^8$–10^9/g feces by day three after inoculation and persisting at that level for ∼15 days (**Figure 7A**, "*Cr*"). Intestinal colonization diminished after day 15 post-infection, but unlike infection of untreated mice, easily detectable CFU were present until the experiment was terminated at 31 days post-infection. Although *C. rodentium*Δ*eae* was present in the stool at high levels at three days post-infection, this strain was not capable of stable colonization (**Figure 7A**, "*Cr*Δ*eae*"). The colonization defect was fully complemented by plasmid-borne EHEC or EPEC intimin for at least the first 15 days post-infection (**Figure 7A**, "*Cr*Δ*eae*/pInt$_{EHEC}$" or "*Cr*Δ*eae*/pInt$_{EPEC}$," respectively). At later time points, complementation was not complete, as bacteria expressing either EPEC or EHEC intimin were cleared somewhat more rapidly than wild type *C. rodentium*. These data indicate even when the flora is disrupted by antibiotic pre-treatment, intimin is required for maximal murine colonization. In addition, the late time point differences notwithstanding, EHEC or EPEC intimin were able to provide wild type colonization function for the first two weeks of infection.

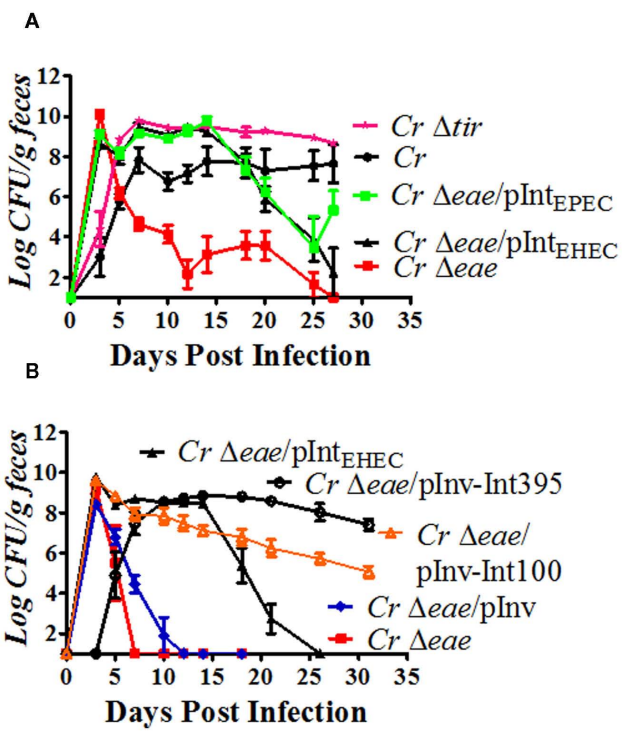

FIGURE 7 | Intimin but not Tir is required for colonization of streptomycin pre-treated mice by *C. rodentium*. (A,B) Eight-week-old C57BL/6 mice were pre-treated with streptomycin in their drinking water for 48 h prior to being inoculated with approximately 5×10^9 CFU of indicated *C. rodentium* strains by oral gavage (see Materials and Methods), with the exception of *C. rodentium*Δ*tir* in **(A)**, in which approximately 10^6 CFU were inoculated. Colonization was determined by viable stool counts, and shown are the averages CFU (±SEM) of three to five mice per group. [Note that the slightly lower level of colonization by *C. rodentium*Δ*tir* in **(A)** at three days post-infection was likely due to the lower inoculum used]. Each strain was tested in at least two independent experiments.

To determine whether the requirement for intimin was a reflection of its ability to interact with Tir, we inoculated streptomycin pre-treated mice with a *C. rodentium*Δ*tir* mutant. Notably, whereas Tir is essential for colonization by *C. rodentium* in conventional mice (Deng et al., 2003), we found that Tir was dispensable in streptomycin pre-treated mice. In fact, fecal counts of *C. rodentium*Δ*tir* reached approximately 10^{10} CFU/gram of feces by six days post-infection and remained highly elevated thereafter (**Figure 7A**, "*Cr*Δ*tir*"). Clearly, intimin provides a Tir-independent colonization function in this infection model.

To test whether the potential integrin binding activity of intimin might be the Tir-independent activity that provides this function, we infected streptomycin pre-treated mice with *C. rodentium*Δ*eae* harboring pInv, which expresses the β_1-chain integrin ligand invasin. *C. rodentium*Δ*eae*/pInv, like *C. rodentium*Δ*eae*, was present at high levels transiently (at post-infection day three) in the stool of infected mice but was typically unable to sustain infection, suggesting that integrin binding of intimin did not contribute to colonization function (**Figure 7B**, "*Cr*Δ*eae*/pInv"). (As was the case for infection of untreated C57BL/6 mice, occasionally the expression of invasin promoted a level colonization several orders of magnitude lower than *C. rodentium* that expressed endogenous or EHEC intimin; data not shown).

Finally, to determine what region of intimin was required to promote infection of antibiotic pre-treated mice, we infected mice with a *C. rodentium*Δ*eae*/pInv–Int395 or *C. rodentium*Δ*eae*/pInv–Int100. We found that expression of either Inv–Int395, which binds Tir, or Inv–Int100, which does not, was sufficient to promote stable high level colonization (**Figure 7B**, "*Cr*Δ*eae*/pInv–Int395" and "*Cr*Δ*eae*/pInv–Int100"). In some instances, the degree of colonization by *C. rodentium*Δ*eae*/pInv–Int100 was somewhat less than *C. rodentium*Δ*eae*/pInv–Int395 after ten days post-infection, but remained $>\sim 10^6$/g feces throughout the 25- to 31-days of infection (**Figure 7B** and data not shown). Thus, it appears that the C-terminal 100 residues of intimin contain the activity that promotes colonization of antibiotic pre-treated mice. The finding that this portion of intimin is incapable of binding to Tir is consistent with the hypothesis that a Tir-independent function of intimin provides acritical colonization activity in animals with altered flora.

DISCUSSION

Intimin, encoded by *eae*, is an adhesin essential for colonization by AE pathogens (Donnenberg et al., 1993a; Schauer and Falkow, 1993b; Tzipori et al., 1995; Dean-Nystrom et al., 1998; Ritchie

et al., 2003). Allelic variation of intimin has been associated with differences in colonization of intestinal samples *ex vivo* (Fitzhenry et al., 2002; Mundy et al., 2007), and inoculation of gnotobiotic piglets with an EHEC strain harboring a plasmid encoding EPEC intimin resulted in colonization of the small intestine, a property not possessed by the same strain harboring a plasmid encoding EHEC intimin (Tzipori et al., 1995). To avoid potential effects related solely to high copy number expression of intimin, in this study we precisely replaced the endogenous (chromosomal) *eae* coding sequence of EHEC with EPEC *eae*. In fact, we found that the recombinant strain was incapable of efficiently colonizing the small intestine and infected the piglet indistinguishably from its isogenic wild type EHEC parent. A previous study utilizing a newborn (not gnotobiotic) piglet model also failed to note differences in tissue tropism due to intimin allele, although that study was not designed to carefully assess tissue tropism (Donnenberg et al., 1993a). Preliminary findings suggest that neither overexpression of EHEC nor EPEC intimin is associated with clear change in EHEC tissue tropism in gnotobiotic piglets, suggesting that simple overexpression does not account for the previously described tropism differences (Michael J. Brady and Saul Tzipori, unpublished observations).

To assess potential functional differences specific to intimin to particular alleles in a second animal model, we infected mice with *C. rodentium* strains expressing endogenous intimin, or intimin of canonical EPEC or EHEC strains. We found that EHEC intimin, like EPEC intimin, promoted efficient intestinal colonization and wild type disease. Notably, previous studies indicated that *C. rodentium* expressing a chimeric intimin composed of the 554 N-terminal residues of EPEC intimin fused to the 380 C-terminal residues of EHEC intimin colonized the murine intestine approximately a 100-fold less efficiently than wild type *C. rodentium* and was entirely defective for inducing disease (Hartland et al., 2000; Mundy et al., 2007). The N-terminal 554 residues of EPEC and EHEC intimin are 97% identical, and although we cannot rule out the possibility that an important (perhaps Tir-binding) activity of EHEC intimin is diminished by the exchange of its N-terminus for that of EPEC intimin, an alternative hypothesis is that differences in the murine host strain contribute to the apparent discrepant results; previous studies demonstrating allele-specific differences utilized Swiss NIH or C3H/Hej mice (Hartland et al., 2000; Mundy et al., 2007), whereas we utilized C57BL/6 mice in the current study. Additionally, anecdotal evidence suggests that the degree of murine infection by *C. rodentium* is dependent on the commercial source of mice (M. McBee, personal communication), a factor that might influence the composition of the intestinal microbiome. Regardless, the demonstration here that EHEC intimin can confer apparently full biological function in this infection model provides a facile experimental system in which to assess either EHEC intimin function or therapeutic strategies targeting this important virulence factor.

Intimin has the capacity to recognize mammalian proteins, such as β_1-chain integrins or nucleolin, activities that have been postulated to facilitate colonization by promoting initial bacterial attachment to mucosal surfaces (Frankel et al., 1996a; Sinclair and O'Brien, 2002). Expression of invasin, a high affinity β_1-chain integrin ligand, conferred on *C. rodentium* the ability to enter mammalian cells *in vitro*, indicating that the invasin integrin binding domain was presented in a functional form on the bacterial surface. Invasin did not, however, provide consistent intimin function in promoting murine colonization by *C. rodentium*, indicating that the β_1-chain integrin binding activity is not the sole activity of intimin required for biological function.

The above finding is predicted from the demonstration that an essential receptor for intimin is the type III effector Tir, which becomes localized in the plasma membrane after translocation (Kenny et al., 1997). Tir-binding by intimin is required for high level attachment to cultured mammalian cells (Liu et al., 1999), and Tir-deficient mutants of AE pathogens, like intimin mutants, are incapable of intestinal colonization (Marches et al., 2000; Deng et al., 2003; Ritchie et al., 2003). We found that *C. rodentium* expressing EHEC intimin was capable of Tir-mediated binding to mammalian cells. A *C. rodentium* strain expressing the intimin–invasin chimera Inv–Int395, which contains three intimin immunoglobulin-like domains and the C type lectin-like domain (Frankel et al., 1995; Luo et al., 2000), also efficiently bound to mammalian cells. *C. rodentium* expressing Inv–Int181, which contains a region that is capable of binding Tir when expressed as a recombinant protein, did not bind primed cells or unmanipulated cells. Previous analysis of recombinant intimin derivatives revealed that sequences N-terminal to the minimal 181-residue Tir-recognition domain may influence binding (Liu et al., 1999). Interestingly however, a laboratory *E. coli* K12 strain expressing Inv–Int181, in contrast to *C. rodentium* expressing this chimera, bound to mammalian cells that had been pre-infected with EPEC (Liu et al., 1999). LPS O antigen can sterically hinder the recognition of invasin in the outer membrane (Voorhis et al., 1991), and the strain-specific difference in the activity of Inv–Int181 may reflect differences in O antigen length and/or density.

Intimin-mediated clustering of Tir in the plasma membrane promotes the formation of an actin assembly complex beneath bound bacteria, leading to actin assembly (Campellone et al., 2004a; Touze et al., 2004). Indeed, *C. rodentium* expressing EHEC intimin not only bound to monolayers that were primed with EPECΔ*eae*, but also triggered robust actin assembly. In contrast, whereas *C. rodentium* expressing Inv–Int395 was capable of Tir-mediated binding to mammalian monolayers, this interaction did not trigger efficient pedestal formation. Given the extensive sequence differences between invasin and intimin, any number of factors may account for the partial function of Inv–Int395 in pedestal formation. However, multimeric interactions greatly facilitate the efficiency of actin assembly (Blasutig et al., 2008; Padrick et al., 2008; Sallee et al., 2008), and it is possible that Tir-binding by *C. rodentium* producing Inv–Int395 did not result in the high-density membrane clustering of Tir that triggers efficient actin assembly. Consistent with this hypothesis, *C. rodentium* expressing Inv–Int395 generated foci of Tir in infected cells at a significantly lower efficiency did *C. rodentium* expressing full-length *C. rodentium*, EPEC, or EHEC intimin. Indeed, whereas latex beads coated at high concentrations with C-terminal fragments of purified EHEC intimin stimulated robust actin assembly on monolayers pre-infected with EPECΔ*eae*, *E.*

coli K12 strains expressing invasin–intimin hybrids containing the equivalent regions of intimin did not (Liu et al., 1999).

The finding that *C. rodentium* expressing either EHEC intimin or Inv–Int395 both bound to Tir but only the former triggered actin pedestal formation *in vitro,* provided an opportunity to determine if pedestal formation correlated with intestinal colonization. Indeed, whereas *C. rodentium* expressing EHEC intimin was capable of colonizing mice to levels indistinguishable from levels attained by wild type *C. rodentium*, *C. rodentium* expressing Inv–Int395 colonized mice at levels a million-fold lower, barely above background, and were cleared from the mice by day five post-infection. *C. rodentium*Δ*eae* expressing Inv–Int395 also was not associated with any manifestations of disease. A correlation between the ability to generate robust pedestal *in vitro* and efficient colonization of the mammalian host has previously been described. First, an EHEC strain lacking a second translocated effector, $EspF_U$ (also known as TccP), remains capable of Tir translocation and intimin-mediated binding but is incapable of stimulating robust actin assembly (Campellone et al., 2004b; Garmendia et al., 2004), and is defective (albeit mildly) in late-stage colonization in infant rabbits (Ritchie et al., 2008). Second, a *C. rodentium* encoding a mutant Tir that binds intimin but is deficient in downstream actin signaling is out-competed late in infection by a wild type strain during co-infection experiments (Crepin et al., 2010). Thus, mutants of any of the three bacterial factors directly involved in pedestal formation, intimin, Tir, and $EspF_U$, that specifically diminish pedestal formation *in vitro* also diminish colonization *in vivo*. A genetically engineered *C. rodentium* strain that generates pedestals using an additional mechanism did not display a competitive advantage over a wild type strain (Girard et al., 2009), suggesting that even though inefficient pedestal formation is associated with diminished colonization, pedestal formation enhanced over wild type levels does not lead to enhanced colonization. The means by which a threshold pedestal formation activity may be required for maximal intestinal colonization is currently unclear, but the observation that the EHECΔ*espF*$_U$ mutant formed smaller than wild type aggregates on the intestinal epithelium of piglets and showed a late colonization defect in infant rabbits (Ritchie et al., 2008) suggests that pedestals may stabilize bacterial interaction with the mucosal surface or otherwise promote expansion of the infectious niche at that site.

Whereas Tir-mediated actin pedestal formation appears to contribute to colonization of gnotobiotic piglets and conventional mice, infection of streptomycin pre-treated revealed a Tir-independent function of intimin. Intimin function in this model was largely independent of intimin allele – *C. rodentium* expressing EPEC or EHEC intimin colonized these mice at high levels for at least two weeks, although they both showed somewhat diminished colonization relative to wild type *C. rodentium* beyond that time point. Most notably, whereas *C. rodentium*Δ*eae* was incapable of stably colonizing antibiotic-treated mice, *C. rodentium*Δ*tir* colonized these mice at least as well as wild type *C. rodentium*. The ability of intimin to bind host cells or components of host cells such as β_1-chain integrins or nucleolin has been previously mapped to C-terminal portions of intimin (Frankel et al., 1994, 1996a; Sinclair and O'Brien, 2002, 2004). We found that the colonization activity of intimin in this infection model was retained by intimin derivative sharboring C-terminal regions of intimin, even if such derivatives (e.g., Inv–Int100, containing only the C-terminal 100 residues of intimin) were incapable of binding Tir. Intimin also contributes to the disruption of epithelial barrier function *in vitro* in a Tir-independent manner (Dean and Kenny, 2004). Notably, the Tir-independent intimin activities in cell attachment or barrier disruption have been characterized entirely by *in vitro* assays, and the contribution of these activities to colonization and disease during mammalian infection has not been documented. A comparison of these Tir-independent intimin *in vitro* activities to intimin function in promoting colonization of streptomycin-treated mice might provide insight into novel activities of intimin relevant to colonization of a mammalian host.

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