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Mallick EM, Brady MJ, Luperchio SA, Vanguri VK, Magoun L, Liu H, Sheppard BJ, Mukherjee J, Donohue-Rolfe A, Tzipori S, Leong JM, Schauer DB. (2012). Allele- and tir-independent functions of intimin in diverse animal infection models. Morningside Graduate School of Biomedical Sciences Student Publications. https://doi.org/10.3389/fmicb.2012.00011. Retrieved from https://escholarship.umassmed.edu/gsbs_sp/1780

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

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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 enteroocyte effacement (LEE), a pathogenicity
was found to be essential for colonization of conventional mice, whereas the ability to trigger Tir-mediated pedestal formation differences in intimin in two animal infection models. Notably, abilities. Colonization was not detectably altered by allele-specific or a set of EHEC intimin derivatives with varying Tir-binding 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 β1-chain integrins (Isberg et al., 1987), and EPEC intimin was shown to be capable of recognizing β1-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 differences in species host range, because whereas *C. rodentium* (Tzipori et al., 1995). Allelic differences may also contribute to differences in 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 Bio-analytical, Natick, MA, USA) with 50% glycerol at either −80°C 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 1000, 75μg/ml respectively. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2.

#### CONSTRUCTION OF pCVD438 (pINTePEC) DERIVATIVES

Genomic DNA from EHEC O157:H7 strain EDL933 and plasmid pINTePEC were purified using standard methodologies. All primers used to construct these pCVD438 derivatives are listed in Table 3. To construct pHL69 (pINTeHEC), 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* open reading frame (ORF) in pINTePEC (from the *HindIII* site to the *eae* start codon), the region 3′ of the *eae* ORF in pINTePEC (from the *eae* stop codon to the *SalI* site), and the coding region of the *eae* gene from EDL933. To generate the first two fragments, pINTePEC was restriction enzyme digested with *BsiEI* to delete an internal 1,847-bp fragment (nucleotides 585–2432 of *eae*) and used as the PCR template. The coding region of EDL933 was amplified from genomic DNA. Each primer at a junction point in the fusion PCR was tagged 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 pACYC184 primers listed in Table 3. The fusion PCR product was confirmed by DNA sequencing and ligated into the corresponding *HindIII*/SalI site of pINTePEC, effectively replacing the *eae* ORF of pINTePEC with that of EHEC EDL933. The ligated 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 plnV, plnV–Int395, plnV–Int181, and plnV–Int100 (Table 2). In the case of plnV, the coding region of the *Y. pseudotuberculosis* *inv* gene was amplified from pRL203. 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, pHLS3, pHLAG, and pHLS5, respectively, were used as template DNA to amplify the coding region for plnV–Int395, plnV–Int181, and plnV–Int100, respectively.

#### GENERATION OF C. RODENTIUM STRAINS

Plasmid DNA was isolated using the QIAquick 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*, KanR | 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 |
| DHelx | Fe80DlacZΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17 l/r− mK+ relA1 gal− deoR thi-1 phoA supE44 lrr− A96 relA1 gal− | Gibco BRL |
| SAL35  | DHelx/pHL70Fe80DlacZΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17 l/r− mK+ relA1 gal− deoR thi-1 phoA supE44 lrr− A96 relA1 gal− | This study |
| EDL933 | EHEC O157:H7 | Riley et al. (1983) |
| JPN15.96| EPEC eae::TnphoA | Jerse et al. (1990) |
| BL21 DE3/pDV205 | EHEC Tir | Vingadassalom et al. (2009) |
| TUV93-0 | EHEC TUV93-0 Δeae | Murphy and Campellone (2003) |
| KM60  | EHEC TUV93-0 Δeae | Murphy and Campellone (2003) |
| KM48  | EHEC TUV93-0 Δeae::cat-sacB | Murphy and Campellone (2003) |
| JPN15/pMAR7 | AmpR derivative of E2348/69 O127:H6 prototype | Jerse et al. (1990) |
| LM-1   | KM46 derivative expressing EHEC tir-cesT and EPEC eae | This study |
| C. rodentium Δtir | DBS100 with an in-frame deletion of tir; ZeoR (i.e., zeocin resistance) | This study |

Table 2 | Description of plasmids used in study.

| Plasmid | Protein | Description | Reference |
|---------|---------|-------------|-----------|
| pCVD438 | IntEPEC | pACYC184-derived plasmid encoding eae from E2348/69 | Donnenberg and Kaper (1991) |
| pHL69  | IntEPEC | pCVD438 with eaeEPEC ORF replaced by eaeEHEC ORF | This study |
| pHL70  | Inv    | pCVD438 with eaeEPEC ORF replaced by inv ORF | This study |
| pHL76  | Inv−Int395 | pCVD438 with eaeEPEC ORF replaced by the 5′ 489 codons of inv fused to the 3′ 395 codons of eaeEHEC. | This study |
| pHL79  | Inv−Int181 | pCVD438 with eaeEPEC ORF replaced by the 5′ 793 codons of inv fused to the 3′ 181 codons of eaeEHEC. | This study |
| pHL80  | Inv−Int100 | pCVD438 with eaeEPEC ORF replaced by the 5′ 878 codons of inv fused to the 3′ 100 codons of eaeEHEC. | This study |
| pT7-4  | AmpR, P410 | pT7-4 with inv from *Y. pseudotuberculosis* | Tabor and Richardson (1985) |
| pRI203 | pT7-4 | cloning vector, CamR, TetR | Isberg et al. (1987) |
| pACYC184 | pACYC184 | cloning vector, CamR, TetR | Rose (1988) |
| pHL35  | pT7-4 with inv (bp 1–1467)/eaeEHEC (bp 1617–2802); produces the protein Inv−Int395 | Liu et al. (1999) |
| pHL49  | pT7-4 with inv (bp 1–2379)/eaeEHEC (bp 2260–2802); produces the protein Inv−Int181 | Liu et al. (1999) |
| pHL55  | pT7-4 with inv (bp 1–2634)/eaeEHEC (bp 2503–2802); produces the protein Inv−Int100 | Liu et al. (1999) |
| pDV205 | EHEC Tir | pET21 containing EHEC tir | Vingadassalom et al. (2009) |
| pUC19  | pUC19 | High copy cloning vector, AmpR | Yanisch-Perron et al. (1985) |
| pHL4   | IntEPEC | pUC19 derivative encoding EPEC eae | Liu et al. (1999) |
| pHL6   | IntEPEC | pUC19 derivative encoding EHEC eae | Liu et al. (1999) |
| pTP223 | IntEPEC | Produces λ-garnet-exa downstream of lac promoter | Murphy and Campellone (2003) |
| pKD46  | IntEPEC | Expresses lambda-red recombinase, AmpR | Datsenko and Wanner (2000) |

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.

| Primera | Sequence |
|---------|----------|
| **eaeEPEC 5 REGIONb,c** | |
| Fp-pACYC184 | 5’ acc tga agt cag ccc cat acg ata 3’ |
| R-eaeEPEC | 5’ agt aat cat gtt tgg gct cca cca cag tgg 3’ |
| R-eaeEPEC-2 | 5’ aac cat cat gtt tgg gct cca cca cag tgg 3’ |
| **eaeEPEC 3 REGIONb,d** | |
| F-eaeEPEC | 5’ gtt tgt gta gaa ta 3’ |
| R-pACYC184 | 5’ ccc ctc gac ggt ctc tct tat ggc act cc 3’ |
| F-eaeEPEC-2 | 5’ ggc gct tca ata ta 3’ |
| R-pACYC184-2 | 5’ tga ctt cct gtc agg ctc tca agg 3’ |
| **OPEN READING FRAMESb,e** | |
| F-eaeEHEC | 5’ aag cca aac atg atc acct gtt ggt ta 3’ |
| R-eaeEHEC | 5’ aag tat tta ata atc ttc tac aca aac cgc at 3’ |
| F-inv | 5’ aag cca aac atg atg ttc cag cca at 3’ |
| R-inv | 5’ aag tat tta ata atc tat tga cag cgc aca ga 3’ |
| **C. RODENTIUM tir MUTANTG** | |
| FA-Tir-KO | 5’ gtt ctc aat cat cca tca gtc ac 3’ |
| R-B-Tir-KO | 5’ acc aag atc cct taa cgt ggg ttc gca cct ctc atg gca aag cga cca cat ata tcc ctt tat tta att atc gga aat ttt aca cta atc c 3’ |
| FC-Tir-KO-zeo | 5’ ggc gct ctg ctc ctc ctc ggc cag cag ctc ggc gcc ggc ggc ggc gtc gc 3’ |
| R-D-Tir-KO-zeo | 5’ tca gtc ctg ctc ctc ctc ggc cag cag ctc ggc gcc ggc ggc ggc gtc gc 3’ |
| FE-Tir-KO | 5’ gtt ctc tgg ggc gct cgc gag cgg cag gag cag gag cgc ctc gtc gag gct cgc gag cgg cgc ggc ggc ggc gtc gc 3’ |
| R-E-Tir-KO | 5’ ggc ccc aac acc atg atg tag 3’ |
| FTI-Ext | 5’ gct tgt atca taa gtt atc ccc at 3’ |
| R-Tir-Ext | 5’ ctt gct cct tag cag cta cag cga ccc at 3’ |
| F-Tir-Int-323–342 | 5’ ttc gtt ttg aac aag acg ca 3’ |
| R-Tir-Int-819–938 | 5’ tgc gct ctt att ctt cag ctt cat ttt 3’ |
| F-Tir-Int-590–610 | 5’ ata cag gtg ctt tgg tgg cct 3’ |
| R-Tir-Int-1049–1069 | 5’ cca att cct gac gtt gta 3’ |
| F-Zeo-Int | 5’ gag cgg tgg aag tgt ttt tgg gca 3’ |
| R-Zeo-Int | 5’ ggc aag cgg tgc acg cag t t 3’ |

* aF forward (top strand) primer; R, reverse (bottom strand) primer.
* bUnderlined sequences represent the restriction enzyme sites used for cloning. Italicized sequences indicate the tails used for fusing eaeEPEC 5 and 3 sequences with eaeEPEC INV, or hybrid allele cloning regions.

Table 3 continued...

dae (~2.8 kb) with 3’ EHEC tail, and EHEC 3’UTR (~960 bp). The PCR product was cloned into pGEM7xf(+), linearized by BamHI and XbaI digestion, and transformed into JM109 harboring pT7223. Transformants were screened by sucrose resistance and chloramphenicol sensitivity. Candidates were confirmed by PCR and sequencing.

**GENERATION OF C. RODENTIUM ΔTIR**

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 from Promega, Madison, WI, USA) as a template. Primers F-C-Tir-KO-zeo and R-D-Tir-KO-zeo were used to amplify a 504-bp PCR product 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-390–410, 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 (ampR) 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). Mice were sacrificed on day 38 and blood was harvested via cardiac bleed, allowed to clot at room temperature for 30 min, and the 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% CO₂ 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% CO₂ 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% CO₂ 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% CO₂ 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 Δeae, or C. rodentium Δeae 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°C in 5% CO₂. 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 (Diagnostic 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 (+/− antibotic) for 8 h. Cultures were diluted 1:500 into 5 ml DMEM supplemented with 0.1 M HEPES (pH 7.0; +/− antibotic) and incubated at 37°C without agitation with 5% CO₂ 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% CO₂. 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% CO₂. For infections, cell monolayers were washed twice with sterile PBS following 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% CO₂ 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, and maximum number of bacteria bound to cells was determined. The stratification of the number of cells with the given amount of 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 antisera (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 EHECΔeae 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 ~2 × 10⁹ 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 Macokey 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 DH₂O 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.
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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 EHECΔeae 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. rodentiumΔeae mutant for colonization and intestinal disease in mice. Previous work demonstrated that C. rodentiumΔeae harboring pCVD438, which encodes EPEC intimin, was fully virulent in Swiss NIH and C3H/HeJ mice (Hartland et al., 2000; Mundy

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    | +/−          |
| EHECΔeae      | 4              | 0    | −            |
| EHEC LM-1     | 4              | 4    | +/−          |

Gnotobiotic piglets were infected orally with 5 × 10⁹ EHEC, EHECΔeae, 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.

![Figure 1](http://www.frontiersin.org)
et al., 2007). In contrast, C. rodentiumΔeae 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 “pIntEPEC”) with the EHEC eae coding sequence to create pIntEHEC (i.e., pHL69; Table 2). C57BL/6 mice were infected with approximately 5 × 10⁹ CFU of C. rodentium, C. rodentiumΔeae, C. rodentiumΔeae/pIntEPEC, or C. rodentiumΔeae/pIntEHEC. Mice infected with C. rodentium reached peak colonization levels seven days post-infection with approximately 10⁹ 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. rodentiumΔeae in the stool peaked at three days post-infection, reaching less than 10⁸ bacteria per gram of feces, and quickly diminished, never rising to more than 10⁴ 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. rodentiumΔeae promoted high level colonization with comparable kinetics to wild type C. rodentium (Figure 2A). Additionally, intestinal sections from rodents infected with C. rodentiumΔeae 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. rodentiumΔeae, promoted colonization, colonic hyperplasia, and intestinal damage indistinguishable from wild type C. rodentium (Figure 2) and generated AE lesions morphologically indistinguishable from 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. rodentiumΔeae 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 EPECΔeae 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. rodentiumΔeae/pIntEHEC bound to pre-infected monolayers indistinguishably from that of wild type C. rodentium, and approximately 20-fold more efficiently than C. rodentiumΔeae (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 β₁-chain integrins (Isberg and Leong, 1990; Frankel et al., 1996a), was previously used to deliver various...
of EHEC intimin to the surface of the bacteria in the form of invasin–intimin hybrids (Liu et al., 1999). C. rodentiumΔeae 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. rodentiumΔeae 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. rodentiumΔeae, 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 EPECΔeae 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. rodentiumΔeae 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). Invasin was required for binding, because approximately 20 of the 25 cells infected with C. rodentiumΔeae were bacteria-free, and virtually no cells harbored more than five bacteria (Figure 4B). EHEC intimin, when expressed in C. rodentiumΔeae, 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. rodentiumΔeae, dramatically enhanced the ability of the bacteria to associate with cells (p < 0.03), 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. rodentiumΔeae 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. rodentiumΔeae, 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. rodentiumΔeae 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. rodentiumΔeae 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, “Gr”). 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, “pPlntEHEC” and “pPlntEPEC”, 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. rodentiumΔeae expressing invasin–intimin hybrids. C. rodentiumΔeae 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. rodentiumΔeae (Figure 5, “pInv”, “pInv–Int181”, or “pInv–Int100”). In contrast, 59% of C. rodentiumΔeae expressing Inv–Int395, which

**FIGURE 3** | Enterohemorrhagic *E. coli* intimin can complement a C. rodentiumΔeae 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. rodentiumΔeae/plmtΔeae. Original magnification, 25,000×. Bar, 0.5 μm.
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*Δeae and significantly lower than that of wild type *C. rodentium* or *C. rodentium*Δeae 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*Δeae mutant for colonization and disease *in vivo*. As shown above (Figure 2A), after oral gavage of C57BL/6 mice with approximately 5 × 10⁸ *C. rodentium*Δeae/pInvEHEC, *C. rodentium*Δeae 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*Δeae/pInv displayed colonization kinetics indistinguishable from *C. rodentium*Δeae (Figure 6A) and did not trigger colitis (Figure 6B), showing that bacterial binding to β₁-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*Δeae/pInv colonized mice somewhat better than *C. rodentium*Δeae, 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*Δeae 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*Δeae, and resulted in no detectable intestinal histopathology (Figures 6A,B). Notably, *C. rodentium*Δeae 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*Δeae in colonization and triggered...
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. rodentiumΔeae 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. rodentiumΔeae. # Indicates that percent positive bacteria is significantly (*p < 0.05) lower than that for C. rodentium/pIntEHEC.

ATir-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. rodentiumΔeae/pInv and C. rodentiumΔeae 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* post-infection, this strain was not capable of stable colonization of conventional mice (Deng et al., 2003), we found that Tir was dispensable in streptomycin pre-treated mice. In fact, fecal counts of *C. rodentium*Δtir approached approximately 10^12 CFU/gram of feces by six days post-infection and remained highly elevated thereafter (*Figure 7A, "CrΔtir"). Clearly, intimin provides a Tir-independent colonization function in this infection model.

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*Δtir 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*Δtir were reduced by approximately 10^6 CFU/gram of feces by six days post-infection and remained elevated thereafter (*Figure 7A, "CrΔtir"). Clearly, intimin provides a Tir-independent colonization function in this infection model.

Finally, to determine which region of intimin binding activity of intimin might be the Tir-independent activity that provides this function, we infected streptomycin pre-treated mice with *C. rodentium*Δeae harboring plnE, which expresses the β1-chain integrin ligand invasin. *C. rodentium*Δeae/plnE, like *C. rodentium*Δeae, 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 an integrin binding of intimin did not contribute to colonization function (*Figure 7B, "CrΔeae/plnE"). (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). In other cases, the degree of colonization by *C. rodentium*Δeae/plnE was somewhat less than *C. rodentium*Δeae/plnE–Int100. In some instances, the degree of colonization by *C. rodentium*Δeae/plnE–Int100 was somewhat less than *C. rodentium*Δeae/plnE–Int395 after ten days post-infection, but remained >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., 2008). 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*Δeae harboring plnE, which expresses the β1-chain integrin ligand invasin. *C. rodentium*Δeae/plnE, like *C. rodentium*Δeae, 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, "CrΔeae/plnE"). (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).

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Finally, to determine which region of intimin binding activity of intimin might be the Tir-independent activity that provides this function, we infected streptomycin pre-treated mice with *C. rodentium*Δeae harboring plnE, which expresses the β1-chain integrin ligand invasin. *C. rodentium*Δeae/plnE, like *C. rodentium*Δeae, 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, "CrΔeae/plnE"). (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).

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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. McBe, 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 β1-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 β1-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 β1-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 stericly 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 EPECΔeae, 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 EPECΔeae, 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. rodentiumΔace 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, EspF1 (also known as TccP), remains capable of Tir translocation and intimin-mediated binding but is incapable of stimulating robust actin pedestal assembly (Campellone et al., 2004b; Gar mendia 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 EspF1, 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 EHECΔespF1 mutant formed smaller than wild type aggregates on the intesti nal 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. rodentiumΔace was incapable of stably colonizing antibiotic-treated mice, C. rodentiumΔtir 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 β1-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 harboring 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.

ACKNOWLEDGMENTS

We thank Joseph Newman for helpful discussion and technical assistance. We thank the UMASS DERC core facility for fixing, sectioning, and staining all tissue sections and also Lara Straitmatter and Greg Hendricks of the UMass Electron Microscopy Core Facility. The anti-EHEC intimin antibody was a generous gift from Alison O’Brien and the anti-OmpA antibody was a kind gift from Carol Kumamoto. This work was supported by PHS grant CA63112 from the NCI to David B. Schauer and R01-AI46454 to John M. Leong. Steven A. Luperchio was supported by NIAMS Training Grant ES07020.

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Wadsworth et al. (2012) Allele- and Tir-independent intimin function in diverse models of virulence. Front. Microbiol. 3, 100211.

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