INTRODUCTION

Enteropathogenic Escherichia coli (EPEC) and enterohaemorrhagic E. coli (EHEC) are important causes of acute gastroenteritis in humans (Nataro & Kaper, 1998). EPEC is a frequent cause of infantile diarrhoea in the developing world (Chen & Frankel, 2005) while EHEC causes a wide spectrum of illnesses ranging from mild diarrhoea to severe diseases, such as haemorrhagic colitis and haemolytic uraemic syndrome (HUS). HUS is the leading cause of acute renal failure in children, and is associated with the production of potent Shiga toxins (Stx) (Thorpe, 2004). Strains of EHEC belonging to serogroup O157 are most commonly associated with severe human disease (Mead et al., 1999). However, infections with other EHEC strains, such as those of serogroups O26 and O103, are on the rise (Brooks et al., 2005).

EHEC and EPEC exhibit narrow host specificity and, given that mice are by and large resistant to symptomatic infection, a difficulty with studying EPEC and EHEC pathogenesis is the lack of a simple small animal model to simulate an in vivo situation. For this reason, infection of mice with Citrobacter rodentium (CR), a natural mouse pathogen that shares many of its virulence factors and mechanism of colonization with EPEC and EHEC, has become a popular surrogate model for in vivo studies (Mundy et al., 2005).

When adhering to intestinal epithelial cells EPEC, EHEC and CR subvert cytoskeletal processes to produce a histopathological feature known as an attaching and effacing (A/E) lesion (Nataro & Kaper, 1998; Garmendia et al., 2005; Mundy et al., 2005). This is characterized by localized destruction of brush border microvilli and intimate attachment of the bacteria to the plasma membrane of the host epithelial cells. The capacity to form A/E lesions is encoded mainly on a pathogenicity island termed the locus of enterocyte effacement (LEE) (McDaniel et al., 1995), which encodes components of a type III secretion system (Jarvis et al., 1995), chaperones, translocator and effector...
proteins (Garmendia et al., 2005) as well as the outer-membrane adhesin intimin (Jerse et al., 1990).

Intimin, the product of the LEE eae gene, was the first to be associated with A/E lesion formation (Jerse et al., 1990). Analysis of intimin sequences from different EPEC and EHEC strains revealed that while the first ~700 amino acids are highly conserved (over 97% identity), the C-terminal 280 amino acids (Int280) are variable; the active receptor-binding site of intimin resides within the Int280 domain (Frankel et al., 1994). Indeed, when expressed as an N-terminal fusion with carrier proteins, Int280 binds directly to epithelial cells (Frankel et al., 1994) and interacts with nucleolin (Sinclair & O’Brien, 2004) and integrin (Frankel et al., 1996a). Int280 also binds the LEE-encoded effector protein Tir, which connects the extracellular bacterium to the host cell cytoskeleton (Kennedy et al., 1997).

The solution (Kelly et al., 1999) and crystal (Luo et al., 2000) 3D structure of Int280α revealed that the polypeptide comprises a series of three globular modules with a distinct organization. The two domains (D1–2) closest to the bacterial cell surface comprise β-sheet sandwiches and structurally resemble immunoglobulin (Ig)-like folds. A third domain (D3) located at the C-terminal tip of the molecule is formed by the 76 amino acid disulfide loop that shows some structural similarity to C-type lectin. The Cys residues (C860 and C937) forming the disulfide loop are totally conserved among the different intimin types and are required for cell-binding activity (Frankel et al., 1995) and A/E lesion formation (Frankel et al., 1998).

Based on polymorphism within Int280, we reported the existence of several different classes of intimin, the most common of which are α, β and γ (Adu-Bobie et al., 1998). In particular, intimin α is associated with the distinct evolutionary lineage of EPEC known as EPEC-1, intimin γ is associated with EHEC O157 : H7, while intimin β appears to be the most ubiquitous type and is found among human and animal pathogens including EPEC-2, EHEC and CR. A large body of evidence suggests that the different intimin types influence host specificity and tissue tropism (Girard et al., 2005; Phillips & Frankel, 2000; Tzipori et al., 1995). In vivo experiments using gnotobiotic piglets revealed that EHEC O157 : H7, which expresses intimin γ, is associated with extensive colonization and destruction of the large intestinal epithelium while EPEC O127 : H6, which expresses intimin α, colonizes both the small and large intestine (Tzipori et al., 1995). Importantly, complementation of an eae mutant of EHEC O157 : H7 with eae, alters the pattern of colonization so that colonization was seen in the small and large intestine in a similar manner to EPEC (Tzipori et al., 1995). Using human and porcine intestinal in vitro organ culture (IVOC) we have shown that as in the gnotobiotic piglet model, intimin exchanges in both EHEC O157 : H7 and EPEC O127 : H6 resulted in alteration in tissue tropism (Fitzhenry et al., 2002a; Girard et al., 2005; Phillips & Frankel, 2000). In this study we compared the functionality of the different intimin types in vivo and ex vivo and performed further intimin exchange studies evaluating the function of CR intimin β in EPEC and EHEC isolates.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains used in this study were wild-type CR strain ICC169, EPEC O127 : H6 (strain E2348/69), EHEC O157 : H7 strain 85-170 and their eae deletion mutants, strains DBS255 (Schauer & Falkow, 1993), CVD206 (Donnenberg & Kaper, 1991) and ICC170 (Fitzhenry et al., 2002a). The plasmids used in this study are listed in Table 1. Plasmid pCVD438 is a pACYC184 vector harbouring the intimin α gene from E2348/69 (Donnenberg & Kaper, 1991). pICC55 is a derivative of pCVD438 in which the 3' end of the eae gene, encoding the Int280α domains, was substituted with a fragment of eae from EHEC encoding Int280β (Fitzhenry et al., 2002a). Bacteria were grown at 37 °C in either high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) or LB and where appropriate, nalidixic acid, kanamycin and chloramphenicol were added to final concentrations of 100, 50 and 35 μg ml⁻¹ respectively.

**Replacing the Int280α coding region in pCVD438 with CR Int280β.** A schematic representation of the strategy used to replace the Int280α region of pCVD438 with Int280β from CR is shown in Fig. 1. Two unique restriction endonuclease sites located in pCVD438 were used, a conserved SalI site located upstream of the Int280 domain (position 1663 of the eae gene) and an Eagl site located downstream of the TAA stop codon and within the pACYC184 vector plasmid (Frankel et al., 1998). The DNA fragment between the SalI and the 3' end of the eae gene encoding intimin β from CR strain ICC169 was amplified by PCR using a forward primer (CReaerfo2 5'-CGGCGTACACAGAATTATGACAGTCGCG-3') and a reverse primer overlapping the end of the gene and including an Eagl restriction site (CReaerlvEagl 5'-CGGCGTACACAGAATTATGACAGTCCC-3'). The amplified eae fragment, flanked by SalI and Eagl restriction sites, was used to replace the corresponding fragments of pCVD438 as previously described (Frankel et al., 1998) (Fig. 1). Following confirmation by DNA sequencing, the modified plasmid, pCC327, was transformed into the eae deletion mutants of EPEC, EHEC and CR, strains CVD206, 85-170 and DBS255, respectively, by electroporation.

**Table 1.** Plasmids used in this study

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pACYC184 | Cm<sup>+</sup> Tc<sup>+</sup> medium-copy-number cloning vector | New England Biolabs |
| pCVD438 | pACYC184 encoding EPEC intimin α | Donnenberg & Kaper (1991) |
| pICC55 | pCVD438 derivative encoding recombinant intimin γ | Hartland et al. (2000) |
| pICC327 | pCVD438 derivative encoding recombinant intimin β | This study |
Infection of mice. Male, specific-pathogen-free C3H/Hej mice that were 6–8 weeks old were purchased from Harlan Olac (Bicester, UK). All the mice were housed in individual ventilated cages with free access to food and water. Bacteria were grown to stationary phase in LB broth containing the appropriate antibiotic. A 1 ml sample of the broth was centrifuged and the bacterial pellet was resuspended in 2.5 ml PBS. Mice were orally inoculated with 200 μl of the bacterial suspension (approx. $1 \times 10^8$ c.f.u. per mouse) by gavage. The viable count of the inoculum was determined by retrospective plating on LB agar containing appropriate antibiotics. Each bacterial strain was tested in independent experiments at least twice using groups of at least five mice per strain. Stool samples were recovered aseptically at various times after inoculation and the number of viable bacteria (c.f.u.) per g stool was determined by plating samples onto LB agar containing appropriate antibiotics. Mice were euthanised by cervical dislocation 8 days post-challenge. Colon and caecum were removed from each mouse, photographed and weighed after removal of stools. The colons were then homogenized mechanically with a Seward 80 stomacher (Seward, London, UK) and the numbers of viable bacteria per colon were determined by plating onto LB agar containing the appropriate antibiotics.

Statistical analysis. All results are presented as the group mean ± SEM. One-way analysis of variance (ANOVA) was performed to test any differences between strains. Analysis was performed using Minitab Statistical Software, release 10.5 Xtra.

Immunofluorescence staining of frozen tissue. Frozen distal mouse colons were embedded in OCT compound (Sakura) and serial sections of 8 μm were cut with an MTE cryostat (SLEE Technik). Sections were picked up on poly-L-lysine-coated slides and air-dried. After formalin fixation for 10 min, tissue sections were blocked with 0.5 % BSA and 2 % normal goat serum in PBS for 20 min. Slides were incubated in primary antibody (rabbit anti-Tir 1:200 or rabbit anti-CR 1:1000) for 60 min at room temperature, washed and incubated in Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) for 30 min. Actin filaments were stained with Alexa Fluor 647 phalloidin (Molecular Probes). Counterstaining of bacteria and cell nuclei was performed using propidium iodide (Sigma). Sections were analysed with a Radiance 2100 confocal laser scanning microscope equipped with an argon-krypton laser and a red diode (Bio-Rad).

Human in vitro organ culture (IVOC). Tissue was obtained with fully informed parental consent and local ethical committee approval using grasp forceps during routine endoscopic (Fujinon EG/EC-41 paediatric endoscope) investigation of gastrointestinal complaints. Proximal small intestinal mucosal biopsies (patients’ age 72, 103, 132 and 181 months) from the fourth part of the duodenum which appeared macroscopically normal were taken for organ culture experiments. Light microscopy subsequently showed no histological abnormality. IVOC infections were performed as described previously (Hicks et al., 1998). In each experiment an uninoculated sample (to exclude endogenous bacterial adhesion) and a positive control were included. Samples were fixed with 2.5% glutaraldehyde, post-fixed in 1 % aqueous osmium tetroxide and processed for viewing by a JEOL JSM 5300 scanning electron microscope (SEM).

Collection and culture of porcine intestinal IVOC explants. Piglets were cared for in accordance with the Guidelines of the Canadian Council for Animal Care. The porcine intestinal IVOC model was used as previously described (Girard et al., 2005). Briefly, segments of the duodenum, jejunum, ileum, caecum and colon were obtained from colostrum-deprived newborn piglets of a conventional herd. Piglets were tranquillized before being euthanized as described elsewhere (Girard et al., 2005). Explants were inoculated three times at hourly intervals with 50 μl broth culture applied to the mucosal surface, and incubated at 37 °C on a rocker in a 95 % O2/5 % CO2 atmosphere for 8 h. Sample explants were processed for SEM as previously described (Girard et al., 2005).

Histopathology. After culture, porcine explants were rinsed thoroughly in sterile PBS and fixed in 10 % buffered formalin for microscopic examination. Formalin-fixed tissues were processed, paraffin-embedded, sectioned at 5 μm, and stained with haematoxylin, phloxine and safranine (HPS) according to standard techniques. Sections were examined by light microscopy for the presence of adhering bacteria on intestinal cells, as previously described (Girard et al., 2005).

RESULTS AND DISCUSSION

Expression of isogenic intimin types in CR

Intimin (encoded by eaeCR) is essential both for colonization of mice by CR and for the production of transmissible colonic hyperplasia (TMCH) (Schauer & Falkow, 1993). Strain DBS255 (ΔΔΔeaeCR) is completely avirulent; although this phenotype could not be complemented in trans, putting the wild-type eaeCR gene back into the chromosome restored virulence (Schauer & Falkow, 1993). Subsequently a pACYC-borne EPEC eae gene (pCVD438) has been shown to complement strain DBS255, restoring virulence and hyperplasia to infected mice (Frankel et al., 1996b). More recently, the eae region within pCVD438 encoding Int280 was replaced with that of EHEC O157:H7 eae, producing plasmid pICC327 (Hartland et al., 2000). Significantly, strain DBS255(pICC327) was unable to cause hyperplasia in mice (Hartland et al., 2000).

The aim of this study was to perform direct comparisons between the functionalities of Int280x, Int280β and Int280γ in vivo. To this end we replaced the region within pCVD438 encoding Int280x with that of CR encoding Int280β, producing plasmid pICC327 (Fig. 1). Although some differences in total intimin expression were noted in Western blots (data not shown), the three plasmids (pCVD438, pICC55 and pICC327) are isogenic in that intimin expression is driven from the same, natural, eae promoter and the three Int280 domains are presented on the surface from the same intimin platform.

Effect of intimin type on colonization of C3H/Hej mice – host specificity

Mice were challenged orally with $1 \times 10^8$ c.f.u. of the wild-type (wt) strain, the ΔΔΔeaeCR strain DBS255 and DBS255 containing pCVD438 (eae280x), pICC55 (eae280x) or pICC327 (eae280β). Stool samples were collected during the course of the infection and the numbers of c.f.u. per g stool were determined by plating. The wt strain had a growth curve typical of CR infection of C3H/Hej mice; the number of c.f.u. per g stool slowly increased over the first few days post-inoculation (p.i.), peaking at days 6–8. In contrast strain DBS255 was shed in stools only for the first 24 h p.i. (Fig. 2A). Plasmid pICC327 (eae280β) fully complemented strain DBS255, restoring colonization and resulting in wt
levels of c.f.u. shed in stools over the 8 day infection (Fig. 2A). Interestingly, plasmid pCVD438 not only fully complemented strain DBS255 in terms of c.f.u. shed in stools, but it colonized mice more efficiently than the wt strain during the first 48 h of infection (Fig. 2B). This is a reproducible result that we have observed on each of the more than 10 occasions that we have tested this strain in mice. In comparison, strain DBS255(pICC55) had an intermediate colonization phenotype, with 1–2 logs fewer c.f.u. shed in stools over the whole 8 day infection (Fig. 2B).

The mice were euthanased at day 8 p.i. and colons were removed for post mortem examination. This time point was chosen as a number of the infected mice had lost 10–15% of their original body weight and had become almost immobile. The macroscopic appearance of the colons is shown in Fig. 3. Mice infected with DBS255 and DBS255(pICC55) had colons of normal appearance with plenty of dark, well-formed stools, no obvious mucosal thickening and a full caecum. Mice infected with the wt and with strains DBS255(pCVD438) and DBS255(pICC327) all showed visible thickening of the distal colon and only a few pale, diffuse stools. In addition, the caecum was often half-full or entirely empty. The distal 8 cm of colon was washed of stools and weighed to give an indication of degree of hyperplasia (Fig. 4A). Mice infected with DBS255 and DBS255(pICC55) had colon weights indistinguishable from those of uninfected mice (<0.2 g), whereas wt-infected mice and those infected with DBS255(pCVD438) had colons which were nearly double the weight (0.4 g).
Colons from mice infected with DBS255(pICC327) had a mean weight of around 0.3 g, intermediate between wt-infected mice and those infected with DBS255.

The levels of c.f.u. recovered from the colons agreed with those shed in stools, with similarly high levels found in mice infected with wt, DBS255(pCVD438) and DBS255(pICC327); mice infected with DBS255(pICC55) had ~10^4 fewer CR bacteria associated with the washed mucosa. No bacteria were recovered from DBS255-infected mice (Fig. 4B).

**Colonization, protein translocation and A/E lesion formation**

In order to visualize adherent CR bacteria, protein translocation and A/E lesion formation, colonic tissues from infected animals were cryosectioned and processed for immunofluorescence microscopy. Adherent bacteria were confirmed as CR using rabbit CR polyclonal antiserum (Fig. 5A). Tir translocation and A/E lesions were apparent in tissue taken from mice infected with wt CR, DBS255(pICC438) expressing eae280, and DBS255(pICC327) expressing eae280\(_b\) (Fig. 5B–D). In contrast, we could not detect adherent DBS255(pICC55) expressing eae280\(_c\) (Fig. 5E).

These results show that in contrast to a previous report (Schauer & Falkow, 1993) a CR eae280\(_b\) plasmid can

**Fig. 3.** Caeca and colons of mice infected with different CR strains for 8 days. Mice infected with strains DBS255 and DBS255(pICC55) showed a full caecum and well-formed dark stools in the distal colon. In contrast, mice infected with wt, DBS255(pCVD438) and DBS255(pICC327) strains had shorter, thicker colons with a few diffuse watery stools and visible hyperplasia at the distal end. In addition, the caeca in these mice were often half-full or completely empty.

**Fig. 4.** Virulence of CR strains in the mouse colon. (A) The total colon was weighed after the removal of all stools at day 8 post-challenge. Mice infected with DBS255(pCVD438) had colon weights that were not significantly different from those of mice infected with the wt CR. There was no significant difference between the colon weights of mice infected with DBS255 and DBS255(pICC55) and the colon weights of uninfected mice. In contrast, the colon weights of mice infected with strain DBS255(pICC327) were significantly greater than those of uninfected mice (P < 0.001) but still slightly less than those of mice infected with the wt (P < 0.05). (B) Mice infected with the wt strain and with DBS255(pCVD438) and DBS255(pICC327) all had similarly high pathogen burdens (around 10^8–10^9 c.f.u. per colon). In contrast, mice infected with DBS255(pICC55) had significantly lower bacterial loads (around 10^4–10^5 c.f.u. per colon; P < 0.001), although the levels were still higher than those in mice infected with the DBS255 mutant, from which no challenge bacteria were recovered.
complement CRΔeae, restoring colonization and hyperplasia. CR expressing eae280 is more virulent than the wt CR, with higher levels of colonization in the first few days of infection. In contrast, despite being present in stools at a relatively high number, CR expressing eae280 did not establish intimate contact with the epithelium and was unable to induce hyperplasia. Considering that all the CR strains are isogenic, that all possess identical type III secretion systems and EspA filaments, and that Int280 binds Tir CR (Hartland et al., 2000), the attenuated phenotype is likely to reflect the absence of a host-cell-encoded intimin receptor.

In a previous study we showed that CR(pICC55) does not induce hyperplasia (Hartland et al., 2000). However, as colonization was only studied in infected tissue at 12 days p.i., we did not record bacterial shedding at earlier time points. Nevertheless, the current study supports our original conclusion that intimin γ is not functionally equivalent to intimin α or β in the CR model.

**Effect of Int280β on tissue specificity – human IVOC**

Previous studies have shown that exchanging intimins between EPEC O127 : H6 and EHEC O157 : H7 resulted in restriction of EPEC colonization to the Peyer’s patch mucosa of human IVOC and extension of colonization of EHEC to proximal small intestine (Fitzhenry et al., 2002a; Phillips & Frankel, 2000). In this study pICC327 was transformed into EPECΔeae (strain CVD206) and EHECΔeae (strain ICC170) mutants. Both CVD206(pICC327) and ICC170(pICC327) adhered to small intestine on 4/4 and 3/4 occasions, respectively (Fig. 6). No adhesion was seen in the eae-negative controls (0/4) (data not shown), while the positive controls EPEC E2348/69 and CVD206(pCVD438) adhered to small intestinal mucosa 4/4 and 3/4 times, respectively (data not shown). These results show that like intimin α, intimin β can also allow colonization of proximal small intestine by EHEC O157 : H7 while EPEC expressing intimin α or intimin β show similar tissue specificity.
Effect of Int280β on tissue specificity – porcine IVOC

A previous study showed that exchanging intimin α and γ between EPEC O127:H6 and EHEC O157:H7 resulted in restriction of EPEC colonization to the ileal mucosa of porcine IVOC and extension of colonization of EHEC to small intestine (Girard et al., 2005).

In order to determine the functionality of intimin β during infection of porcine IVOC, biopsies taken from different sites were infected with recombinant EPECΔeae and EHECΔeae strains. Observation of HPS-stained sections showed that CVD206(pICC327) adhered to all parts of the small intestine at a comparable level to that seen with E2348/69 (Table 2), whereas only a few sites with adhering bacteria were observed in the caecum and the colon (Table 2). For its part, adherence of ICC170(pICC327) was mostly observed in the jejunum, whereas few sites with adhering bacteria were observed in the ileum, caecum and colon (Table 2). Foci of small to large aggregates of adherent bacteria were observed for CVD206(pICC327) (Fig. 7A), whereas relatively small foci or individual adherent bacteria were observed on epithelial cells for ICC170(pICC327) (Fig. 7B). Loose association of bacteria with the intestinal mucosa of some villi, with no obvious change in associated epithelial cell morphology, was observed for the eae mutants CVD206 and ICC170 (data not shown), as previously described (Girard et al., 2005). SEM analysis of the mucosal surface of whole explants inoculated with CVD206(pICC327) demonstrated typical A/E lesions and gross microvillous elongation in the duodenum, jejunum and ileum, whereas the caecum and colon were more slightly colonized, but still demonstrated some A/E lesions (Fig. 8). On the other hand, explants inoculated with ICC170(pICC327) demonstrated only rare A/E lesions with very localized effacement and no microvillous elongation in the duodenum and jejunum (Fig. 8); in all other intestinal segments examined the bacteria were associated with the epithelial cells in small or large aggregates, with no direct evidence of A/E lesions (Fig. 8).

These results show that while intimin β in EPECΔeae can completely restore colonization of porcine IVOC, the expression of intimin β in EHECΔeae does not restore colonization potential fully. This is unlikely to be due to incompatibility between intiminCR and TirEHEC, as EHEC expressing IntCR was functional during infection of human IVOC (Fig. 6), and Deng et al. (2003) have shown that TirCR and TirEHEC are interchangeable.

| Strain          | Duodenum | Jejunum | Ileum | Caecum | Colon |
|-----------------|----------|---------|-------|--------|-------|
| E2348/69        | 13/13 (100%) | 13/13 (100%) | 11/11 (100%) | 6/6 (100%) | 1/6 (16.7%) |
| CVD206          | 1/11* (9.1%) | 5/10 (50%) | 3/11 (27.3%) | 0/11 (0%) | 0/6 (0%) |
| CVD206(pICC327) | 10/12 (83.3%) | 12/12 (100%) | 12/12 (100%) | 4/12 (33.3%) | 1/12 (8.3%) |
| 85-170          | 0/6 (0%) | 2/6 (33.3%) | 14/15 (93.3%) | 3/6 (50%) | 0/6 (0%) |
| ICC170          | 0/6 (0%) | 0/6 (0%) | 14/18* (77.8%) | 0/6 (0%) | 0/6 (0%) |
| ICC170(pICC327) | 0/11 (0%) | 5/11 (45.5%) | 3/12 (25.0%) | 4/12 (33.3%) | 3/12 (25.0%) |

*Although adhering bacteria were observed on HPS-stained sections, SEM demonstrated no A/E lesions for both Δeae strains CVD206 and ICC170 in all intestinal sites assessed.
These results show, as we have shown before for EPEC O55 (Fitzhenry et al., 2002b), that determination of host and tissue specificity by A/E-lesion-forming E. coli is multi-factorial, involving other bacterial and host determinants, as well as intimin.

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