Expression of Intimin γ from Enterohemorrhagic *Escherichia coli* in *Citrobacter rodentium*

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The carboxy-terminal 280 amino acids (Int280) of the bacterial adhesion molecule intimin include the receptor-binding domain. At least five different types of Int280, designated α , β , γ , δ , and ϵ , have been described based on sequence variation in this region. Importantly, the intimin types are associated with different evolutionary branches and contribute to distinct tissue tropism of intimin-positive bacterial pathogens. In this study we engineered a strain of *Citrobacter rodentium*, which normally displays intimin β , to express intimin γ from enterohemorrhagic *Escherichia coli*. We show that intimin γ binds to the translocated intimin receptor (Tir) from *C. rodentium* and has the ability to produce attaching and effacing lesions on HEp-2 cells. However, *C. rodentium* expressing intimin γ could not colonize orally infected mice or induce mouse colonic hyperplasia. These results suggest that intimin may contribute to host specificity, possibly through its interaction with a receptor on the host cell surface.

Enteropathogenic *Escherichia coli* (EPEC) is an important cause of severe infantile diarrheal disease in many parts of the developing world. EPEC bacteria colonize the small intestinal mucosa and, by subverting intestinal epithelial cell function, produce a characteristic histopathological feature known as the “attaching and effacing” (A/E) lesion (11). The A/E lesion is characterized by localized destruction (effacement) of brush border microvilli, intimate attachment of the bacillus to the host cell membrane, and the formation of an underlying pedestal-like structure in the host cell. Similar lesions have been associated with several other bacterial mucosal pathogens, including enterohemorrhagic *E. coli* (EHEC) (23) and *Citrobacter rodentium* (28). EHEC is a food-borne pathogen of worldwide importance which can cause acute gastroenteritis, hemorrhagic colitis, and hemolytic uremic syndrome (23). *C. rodentium* is the causative agent of transmissible murine colonic hyperplasia (3), a disease of laboratory mice characterized by crypt hyperplasia, epithelial cell proliferation, crypt dilation, mucosal thickening, and the development of an uneven epithelial surface of the descending colon.

The first gene to be associated with A/E activity was *eae*, which encodes the bacterial adhesion molecule intimin (16). Mutational analysis of the *eae* genes of EPEC, EHEC, and *C. rodentium* has shown that intimin is necessary for colonization of the host and disease (7, 16, 29). Using human intestinal organ cultures as an infection model system, intimin has been shown to be essential for colonization of the mucosa and A/E lesion formation (13).

Recently, we and others described five distinct intimin subtypes, intimin α , β , γ , δ , and ϵ , based on sequence variation

within the C-terminal 280-amino-acid receptor-binding domain of the polypeptide (Int280) (1, 25). Intimin α is specifically expressed by a group of EPEC strains, all of which belong to one evolutionary branch of EPEC known as EPEC clone 1 (32), and *Hafnia alvei*. Intimin β is mainly associated with EPEC and EHEC strains belonging to their respective clones 2, *C. rodentium* and rabbit diarrheagenic *E. coli* type 1, while intimin γ is associated with EHEC O157:H7 and EPEC O55:H7 (1). This observation raises the possibility that tissue tropism exhibited by EPEC (colonizing mainly the small bowel) and EHEC O157 (colonizing the large bowel) is intimin related. Intimin exchange studies have been performed in piglets using wild-type EHEC (expressing intimin γ) or EHEC expressing the EPEC-derived intimin α . In conventional animals, no differences were seen in intestinal distribution of the A/E lesions (7). However, in gnotobiotic piglets, EHEC expressing intimin α produced A/E lesions in both the small and large intestines whereas EHEC expressing intimin γ produced lesions only in the large intestine (30). More recently, we have shown, using human intestinal explants, that EHEC O157:H7 expressing intimin γ can colonize and induce A/E lesion only on the follicle-associated epithelium of the Peyer's patch (27). In contrast, EPEC O127:H6 expressing intimin α (strain E2348/69) colonized Peyer's patch as well as proximal and distal small intestinal tissues (27). Importantly, tissue tropism towards Peyer's patches was observed following expression of intimin γ in the EPEC background (26). These results suggest that different intimins may play a role in determining the pattern of colonization and tissue tropism in the host.

We and others have shown that Int280 (from EPEC, EHEC, and *C. rodentium*) can bind directly to uninfected host cells (2, 8). However, intimin can also bind to the bacterial receptor, Tir (EspE), which is translocated by the bacteria into the host cell membrane during infection (5, 18). Recently, the global fold of Int280 α in solution was determined by multidimensional nuclear magnetic resonance (17). The structure shows that Int280 comprises three separate domains, two immuno-

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TABLE 1. Plasmids used in this study

Plasmid	Property	Reference or source
pCVD438	pACYC184 encoding intimin α	6
pCVD444	pUC18 encoding intimin γ	33
pET28a	Vector for expression of His-tagged proteins	Novagen
pICC55	pCVD438 derivative encoding recombinant intimin γ	This study
pICC56	pMALc-2 expressing MBP-Int280 γ	This study
pICC58	pET28a expressing His-Tir-Cr	This study
pMALc-2	Vector for generating MBP fusions	New England Biolabs

globulin-like domains and a C-type lectin-like module. Modelling of other intimin types shows that these proteins possess similar structures, which define a new family of bacterial adhesion molecules.

Oral infection of mice with live wild-type *C. rodentium* (15) or intracolonic inoculation of dead bacteria (14) induces a CD3⁺ and CD4⁺ T-cell infiltration into the colonic lamina propria and a T-helper type 1 immune response. This response is not observed, however, in mice inoculated with bacteria lacking intimin, but is seen in mice inoculated with *C. rodentium* complemented with intimin α from EPEC encoded on pCVD438 (14, 15). In this study, we replaced the receptor-binding domain of intimin α on pCVD438 with its intimin γ homologue (generating plasmid pICC55). This hybrid intimin was expressed in *eae* mutant strains of EPEC (CVD206) (6) and *C. rodentium* (DBS255) (28). Although biologically functional, this form of intimin γ could not restore mouse virulence when expressed in DBS255. These results suggest that the variable receptor-binding domain of intimin may contribute to the species specificity exhibited by *C. rodentium* and by other A/E bacterial pathogens.

FIG. 1. Schematic representation showing the construction of recombinant intimin γ . The N-terminal and receptor binding regions of intimin α and intimin γ are represented by different shades. The percentage amino acid identity of each region of intimin α and intimin γ is given in brackets. The position of the restriction site, *SalI*, used to construct the recombinant intimin is given in nucleotides, and the position corresponding to *SalI* in intimin is represented as a dashed line. Schematic is not to scale.

FIG. 2. Western blot analysis of *C. rodentium* strains expressing intimin α and the hybrid intimin γ . Whole-cell protein preparations were separated by SDS-PAGE, and intimins were detected with polyclonal rabbit antibodies raised to a conserved region of intimin. Lane 1, DBS255; lane 2, DBS255(pCVD438); lane 3, DBS255(pICC55).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study were *E. coli* BL21 and TG1 and wild-type *C. rodentium*, EPEC (strain E2348/69), and their *eae* deletion mutants, strains DBS255 (28) and CVD206 (6), respectively. The plasmids used in this study are listed in Table 1. Plasmid pCVD438 is a pACYC184 vector harboring the intimin α -encoding *eae* gene from EPEC E2348/69 (6). pCVD444 is a pUC18 vector harboring the intimin γ -encoding *eae* gene from EHEC EDL933 (33). Bacteria were grown at 37°C in L broth or Dulbecco's modified Eagle's medium. Where appropriate, chloramphenicol and ampicillin were added to final concentrations of 30 and 100 μ g/ml, respectively.

Construction of hybrid intimin γ and maltose-binding protein (MBP)-Int280 γ fusion protein. A schematic representation of the strategy used to produce the hybrid intimin γ is shown in Fig. 1. In order to replace the receptor-binding domain of intimin α in pCVD438 with the intimin γ homologue, we took advantage of two unique restriction endonuclease sites located in pCVD438, a conserved *SalI* site located upstream of the receptor binding domain (position 1663 of the *eae* gene) and an *EagI* site located downstream to the TAA stop codon and within the pACYC184 vector plasmid (9). The DNA fragment between the *SalI* site and the 3' end of the *eae* gene encoding intimin γ from pCVD444 was amplified by PCR using a forward primer (1606-5' GGC AAT AGC TCT AAC AAT GTA) and an *eae* γ -derived reverse primer overlapping the 3' end of the gene and including an *EagI* restriction endonuclease site (5' CTT ACA TGR AGC ATC AGC ATA ATA GGC TTG), as previously described (8). The amplified *eae* fragment, flanked by *SalI* and *EagI* restriction sites, was used to replace the corresponding fragments of pCVD438 as previously described (9) (Fig. 1). Following confirmation by DNA sequencing, the modified plasmid, pICC55 (Table 1), was transformed into the *eae* deletion mutants of EPEC and *C. rodentium*, strains CVD206 and DBS255, respectively, by electroporation.

The DNA fragment encoding Int280 from pICC55 was amplified by PCR (forward, 5'-GGAATTCATTACTGAGATTAAGGCT-3'; reverse, 5'-CCCAA GCTTTTATTYTACACAA-3') and subcloned into pMALc-2, producing plasmid pICC58, in *E. coli* TG1, as previously described (8). Expression of MBP-Int280 γ was induced by IPTG (isopropyl- β -D-thiogalactopyranoside), and the fusion protein was purified as previously described (8).

Detection of intimin expression by Western blotting and FAS. Expression of the intimin derivatives was determined by Western blotting (4). Briefly, stationary L broth cultures were diluted 1:100 in Dulbecco's modified Eagle's medium and incubated for 3 h at 37°C. An equivalent of a culture at an optical density at 600 nm (OD₆₀₀) of 0.5 was loaded onto sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis (SDS-7.5% PAGE) as described (4). The electrophoresed polypeptides were transferred to a nitrocellulose membrane, and immunodetection of intimin was performed using a universal intimin antiserum raised in rabbits against a conserved intimin fragment (amino acids Gly388 to Lys667) (4) diluted 1:500. Fluorescence actin staining (FAS) test was employed for 3 and 6 h to detect A/E lesion formation on HEP-2 cells as described previously (20, 24).

Preparation of *C. rodentium* His-Tir and gel overlays. The DNA segment encoding Tir in *C. rodentium* (Tir-Cr) was amplified using *C. rodentium* DNA as template (primers: forward, 5'-GAAGATCTATGCCTATTGGTAATCTTG

FIG. 3. Infection of healthy human tissue obtained from terminal ileal Peyer's patch of children with CVD206 carrying various plasmids. (A) CVD206(pCVD438). (B) CVD206(pICC55).

GT; reverse, 5'-GAAGATCTTTAGACGAAACGATGGGATC). The *tir* fragment was sequenced and cloned into the *Bam*HI site of pET28a (generating plasmid pICC56) in *E. coli* BL21 previously described (12). For gel overlays, whole-bacterial-cell protein extract from *E. coli* BL21a(pICC56) expressing Tir-Cr was separated by SDS-PAGE, blotted onto a nitrocellulose membrane as described above, and blocked with 10% skim milk in phosphate-buffered saline (PBS)-0.1% Tween 20 overnight. The nitrocellulose membranes were reacted with either rabbit anti-Tir antibodies (12) or with 5 µg of the purified MBP-Int280 fusion proteins per ml in PBS-0.1% Tween 20 for 2 h and washed twice for 5 min in PBS-0.1% Tween 20. Binding of MBP fusions or Tir antibodies to Tir-Cr was detected with rabbit anti-MBP antiserum (1:2,000 for 1 h) and/or anti-rabbit antibodies conjugated to alkaline phosphatase (1:2,000 for 1 h) (12).

IVOC adhesion assay. Tissue was obtained, with fully informed parental consent and ethics approval, using grasp biopsy forceps during routine endoscopic (Olympus PCF pediatric endoscope) investigation of intestinal disorders. Terminal ileal Peyer's patch tissue was taken from patients from areas showing no endoscopic abnormality. IVOC infection was performed as described previously (13). The assay was terminated at 8 h. Each bacterial strain was examined in

vitro organ cultures (IVOC) on at least three occasions by using tissue from different children.

Challenge of mice with *C. rodenticum*. Mice (Swiss NIH, C3H) were orally inoculated by gavage with *C. rodenticum*, DBS255(pCVD438), DBS255(pICC55), and DBS255. Bacteria were diluted with PBS (pH 7.2) to an OD₆₀₀ of 1.7 and delivered to mice in a volume of 100 µl as previously described (15). Mice were killed at 12 days postchallenge, and tissue was snap frozen in liquid nitrogen and stored at -70°C for further analysis.

Immunohistochemistry. Three-step avidin-peroxidase staining was performed on 5-mm-diameter frozen sections as described previously (15) using monoclonal antibodies 145-2C11 (anti-CD3) and YTS 191 (anti-CD4). Biotin-conjugated rabbit anti-rat immunoglobulin G (IgG) (DAKO, High Wycombe, United Kingdom) and goat anti-hamster IgG (Vector Laboratories, Peterborough, United Kingdom) were used at a 1:50 dilution in Tris-buffered saline (TBS) (pH 7.6) containing 4% (vol/vol) normal mouse serum (Harlan Seralab, Oxon, United Kingdom). Avidin peroxidase (Sigma) was used at a dilution of 1:200 in TBS. A two-step protocol was performed using rabbit anti-intimin antibody (4) together with horseradish peroxidase-conjugated swine anti-rabbit IgG secondary anti-

body. Peroxidase activity was detected with 3,3'-diaminobenzidine-tetra-hydrochloride (DAB; Sigma) in 0.5 mg of Tris-HCl (pH 7.6) per ml containing 0.01% H_2O_2 . Endogenous-peroxidase-containing cells were visualized by incubation of sections with DAB substrate and H_2O_2 alone. The density of positive cells in the lamina propria was determined by image analysis as described previously (15).

Inoculation of dead bacteria and analysis of hyperplasia and cellular infiltrate. Formalin-killed bacteria (5×10^8) were injected into the colon of BALB/c mice treated 15 min previously with 0.1 ml of 50% ethanol to break the mucosal barrier (14). Six days later, mice were killed and the colon was dissected out. The weight of the terminal 4 cm of distal colon was determined, and then the sample was immediately snap frozen in liquid nitrogen for immunohistochemistry. Mucosal thickness (i.e., crypt length) was measured on well-oriented sections of colon by using a calibrated eyepiece graticule as described previously (14). At least five measurements were made per sample, and there were five mice per group. The densities of CD3, CD4, and CD8⁺ cells in the lamina propria were analyzed using a Seescan image analyzer as previously described (15).

RESULTS

Construction of hybrid intimin γ . We have shown previously that wild-type *C. rodentium* (expressing intimin β) and *C. rodentium* expressing the EPEC-derived intimin- α [DBS255 (pCVD438)] colonize and produce A/E lesions only in the descending colon of orally challenged mice (10). In order to investigate the tissue selectivity exhibited by *C. rodentium* expressing intimin γ , we expressed this intimin in DBS255 from the high-copy-number, pUC18-derived plasmid pCVD444 (33). A *cat* cassette (pCVD444*) was inserted into this vector to overcome the natural ampicillin resistance of *C. rodentium* (data not shown). Only low levels of intimin were detected in whole-cell extracts of DBS255(pCVD444*) (data not shown). In order to circumvent poor expression, we replaced the receptor-binding domain of intimin α on pCVD438, which was already shown to mediate high levels of intimin expression on the cell surface of DBS255, with that of intimin γ (see Materials and Methods and Fig. 1) to produce a plasmid, pICC55. Since the amino-terminal 554 amino acids of intimin α encoded by the recombinant *eae* gene on pICC55 are 97% identical to the homologous region of intimin γ (Fig. 1), the hybrid intimin is, for all intents and purposes, intimin γ . The hybrid intimin γ was expressed in DBS255(pICC55) and CVD206 (pICC55), and its biological activity was tested. The isogenic strains DBS255(pCVD438) and CVD206(pCVD438) were used as controls.

Expression of intimin was determined by Western blot analysis of whole-cell lysates prepared from the different DBS255 and CVD206 derivatives by using a universal, broad-spectrum polyclonal intimin antiserum reactive with all the different intimin types (4). Lysates from all the recombinant strains, but not from CVD206 or DBS255, reacted with the antiserum (Fig. 2 and data not shown), indicating that the hybrid intimin γ is expressed in DBS255 at a level comparable to that of intimin α and to those of the recombinant CVD206 strains.

Interaction of CVD206 expressing the hybrid intimin γ with HEp-2 cells and human intestinal IVOC. Before the recombinant DBS255 strain was tested in the mouse model, the hybrid intimin γ was subjected to a number of in vitro biological assays, using CVD206(pICC55), designed to determine the influence of the genetic manipulation on the function of this construct. Firstly, the ability of the hybrid intimin γ to mediate A/E lesion formation on HEp-2 cells was investigated. CVD206(pICC55) adhered to cell monolayers in a localized pattern and produced a FAS-positive reaction, similar to that of CVD206(pCVD438) (data not shown). The result shows that this intimin γ can mediate A/E lesion formation on cultured human epithelial cells.

In previous studies, we have shown that the ability of EPEC to induce A/E lesions on human intestinal IVOC is dependent on surface expression of biologically active intimin (13). To

FIG. 4. Fluorescent actin staining of HEp-2 cell monolayers infected with DBS255(pCVD438) (A) and DBS255(pICC55) (B). Arrows indicate FAS-positive bacteria.

test the ability of the hybrid intimin γ to mediate A/E lesion formation on mucosal surfaces, healthy human tissue obtained from terminal ileal Peyer's patch of children was examined after infection with CVD206(pICC55). Like CVD206 (pCVD438), CVD206(pICC55) was able to form A/E lesions on the human tissue (three out of three incubations) (Fig. 3). No binding was observed with CVD206 only (data not shown and reference 12). These results show that pICC55 encodes a biologically functional intimin that can mediate binding and A/E lesion on mucosal surfaces.

Colonization of the mouse intestine following live oral infections with *C. rodentium*. Following verification of the binding activity of the hybrid intimin γ in EPEC, we confirmed its ability to restore A/E lesion formation on HEp-2 cells to DBS255 and tested mouse virulence of DBS255(pICC55). To test the ability of DBS255(pICC55) to mediate A/E lesion formation on HEp-2 cells, monolayers were infected with DBS255(pCVD438) or DBS255(pICC55) cells for 6 h as previously described (24). Both DBS255(pCVD438) and DBS255 (pICC55) cells adhered poorly to HEp-2 cell monolayers, but FAS-positive bacteria were observed for both strains (Fig. 4).

For virulence assays, mice were killed on day 12 postinfection and colons were examined for signs of bacterial colonization and mucosal hyperplasia by immunohistochemistry. The presence of bacteria adhering to the epithelial surface was visualized by immunohistochemistry using the intimin antibody (4) (Fig. 5). *Citrobacter* bacteria were detected in mice infected with the wild-type *C. rodentium* and DBS255(pCVD438) strains, while no bacteria could be seen in mice infected with DBS255 or DBS255(pICC55) (Fig. 5). When bacteria were present, they

FIG. 5. Intimin staining of *C. rodentium*-infected colonic tissue. DBS255(pCVD438) expressing intimin α is seen colonizing deep down into crypts (A) and wild-type *C. rodentium* expressing intimin β is seen adhering to surface exposed epithelium (B), while no bacteria can be seen following infection with DBS255 cells (C) or DBS255(pICC55) cells expressing the hybrid intimin γ (D).

were observed in close association with enterocytes. However, although adhesion remained tissue specific, the cell specificity of DBS255(pCVD438) appeared to be altered, as there was a more extensive colonization of the crypts, confirming our previous observations (10). Colonization of the epithelium was directly associated with mucosal thickening and crypt hyperplasia. Indeed, the colons from wild-type *C. rodentium*- and DBS255(pCVD438)-infected mice were very similar. Macroscopic thickening of the distal colon was observed in all mice in these groups. Microscopic examination showed massive epithelial cell hyperplasia and CD4 cell infiltrate with an increase in mucosal thickness and crypt length (Fig. 6A and B). The colons of mice challenged with DBS255, DBS255(pICC55), or DBS255(pCVD444*) did not show any histopathological changes, and the tissue was indistinguishable from those of uninfected control mice (Fig. 6C and D).

The hybrid intimin γ binds Tir from *C. rodentium*. The fact that the hybrid intimin γ confers A/E lesion formation activity to DBS255(pICC55) on HEp-2 cells indicates that it binds Tir from *C. rodentium* (Tir-Cr). In order to confirm this experimentally, gel overlay binding assays were used to probe for the interaction of the hybrid intimin γ with Tir-Cr. For this purpose, the DNA fragment encoding Tir-Cr was amplified by

PCR and cloned into the expression vector pET28a (generating plasmid pICC56) in *E. coli* BL21. Partial sequencing of the N- and C-terminal regions of Tir-Cr revealed exact homology with a Tir sequence derived from mouse enteropathogenic *E. coli* (MPEC) (accession no. AB 026719) (22). In parallel, the DNA fragment encoding the carboxy-terminal 280-amino-acid receptor-binding domain of the hybrid intimin γ (Int280 γ) was amplified by PCR and cloned into pMAL-c vector (generating plasmid pICC58) in *E. coli* TG1. Int280 γ was overexpressed and purified as an MBP fusion (MBP-Int280 γ). MBP-Int280 α and MBP were used as positive and negative controls, respectively.

Expression of the recombinant His-Tir-Cr was induced by IPTG. Whole-cell extracts were then blotted, following electrophoresis, onto nitrocellulose membranes, and the immobilized Tir-Cr was allowed to react with either the MBP-Int280 α or MBP-Int280 γ fusion proteins or MBP or with anti-Tir antiserum (12). Binding of the intimin derivatives was detected following further incubations with rabbit anti-MBP and/or alkaline phosphatase-conjugated anti-rabbit antisera. These overlay experiments, although they did not provide a quantitative measure, showed that similarly to anti-Tir antibodies,

FIG. 6. Mucosal thickness in mice 12 days after inoculation with live wild-type *C. rodentium* (A), DBS255(pCVD438) (B), DBS255 (C), and DBS255(pICC55) (D). Note that in panels A and B, the mucosa is thick and hyperplastic with an increase in CD4⁺ cells (arrows), in contrast to panels C and D, in which the colon is thin and essentially normal and there are very few CD4⁺ cells (immunoperoxidase with anti-CD4; magnification, $\times 200$).

both Int280 α and the hybrid Int280 γ could bind Tir-Cr (Fig. 7). No binding was detected using MBP only (data not shown).

Mouse colonic hyperplasia and T-cell infiltration. In a previous study, we showed that intracolonic inoculation of mice with dead *C. rodentium* expressing either intimin β or intimin α induced T-cell infiltration to the lamina propria at the base of the crypts and colonic inflammation and hyperplasia without detectable bacterial colonization. To test for the Tir-indepen-

dent biological function of the hybrid intimin γ in vivo, we investigated T-cell infiltration of the colonic submucosa following inoculation with dead DBS255(pICC55) bacteria. In contrast to inoculation with wild-type and intimin α -expressing *C. rodentium*, no T-cell infiltration or increases in colonic weight or mucosal thickness were detected following infection with *C. rodentium* expressing the hybrid intimin γ or DBS255 (Fig. 8). Taken together, these results show that the hybrid

FIG. 6—Continued.

intimin γ cannot restore mouse virulence to *C. rodenticum* and, unlike intimin α and β , it has no biological activity in the murine situation.

DISCUSSION

Intimin was the first gene product of EPEC to be associated with A/E lesion formation (16). Recently, the *eae* genes from different A/E lesion forming bacterial pathogens were categorized into a family of antigenically distinct intimin types (α , β ,

γ , δ , ϵ) (1, 25). Studies on the different intimins from EPEC, EHEC, and *C. rodenticum* have shown that receptor-binding activity is localized to the C-terminal 280 amino acids (Int280) (8). A number of groups have reported that intimin can bind directly to uninfected host cells (2, 8) and to a receptor encoded by the bacteria, termed Tir, which is translocated into the host cell membrane via a type III secretion system (18). Binding to the host cell but not to Tir is dependent on a disulfide bridge at the carboxy terminus of Int280 (12). However, when expressed on the surface of EPEC, both of these binding

FIG. 7. Gel overlays showing binding of MBP-Int280 fusions to Tir from *C. rodentium* (Tir-Cr). Tir-Cr was expressed as a His-tagged fusion. Induced whole-cell protein preparations were separated by SDS-PAGE and overlaid with different purified MBP-Int280's. Lanes 1, 3, and 5: *E. coli* BL21 carrying pICC56; lanes 2, 4, and 6: *E. coli* BL21 carrying pET28a alone (included as a negative control). Blots were overlaid with anti-Tir antiserum (lanes 1 and 2), MBP-Int280 γ (lanes 3 and 4), or MBP-Int280 α (lanes 5 and 6).

activities of intimin are required for intimate bacterial adhesion and A/E lesion formation.

Recently, the global fold of Int280 α in solution was determined by multidimensional nuclear magnetic resonance (17). The structure shows that Int280 is built from three globular domains: D1 (residues 1 to 91), D2 (residues 93 to 181), and D3 (residues 183 to 280). The first two domains, D1 and D2, although lacking disulfide bonds, resemble the type I set of the Ig super family (IgSF). The IgSF domains in intimin appear to form an articulated linker that most likely extends away from the bacterium surface and confers a highly accessible third domain, D3 (residues 183 to 280), for potential interaction. Despite the lack of significant sequence homology, the topology in Int280 D3 is reminiscent of C-type lectins, a family of proteins responsible for cell surface carbohydrate recognition. These findings imply that carbohydrate recognition may be important for intimin-mediated cell adhesion, which in turn may provide a mechanism for tissue tropism exhibited by different A/E lesion forming bacterial pathogens. Indeed, a recent study by Vanmaele et al. (31) showed that coinoculation of EPEC with Lewis X-bovine serum albumin glycoconjugate caused a decrease in intimin expression by the bacteria. These results are consistent with our previous observations of down regulation of intimin expression following A/E lesion formation (19).

Intimin exchange studies performed in piglets suggest that different intimin types might determine tissue tropism. In these studies, wild-type EHEC bacteria (expressing intimin γ) or EHEC bacteria expressing the EPEC-derived intimin α were used. In conventional animals (7), no differences were seen in the intestinal distribution of the A/E lesions, but in gnotobiotic piglets (30), EHEC expressing intimin α produced A/E lesions in both the small and large intestines whereas EHEC expressing intimin γ produced lesions only in the large intestine. More recently we have shown that intimin contributes to tissue tropism exhibited by EPEC (colonizing all regions of human small intestinal explants) and EHEC (which specifically target the follicle-associated epithelium of the Peyer's patch) (26, 27). The aim of the present study was to extend these investigations further to study the contribution of the intimin types to host (species) specificity.

A difficulty associated with working on EPEC and EHEC is the lack of a small animal model for studying the biological properties of EPEC-associated genes in an in vivo situation. *C. rodentium* causes transmissible colonic hyperplasia in mice

(3), an infection associated with the formation of A/E lesions similar to those described for human EPEC (28). *C. rodentium* has been shown to harbor the loss of enterocyte effacement pathogenicity island encoding an *eae* homologue (21) that directs the expression of an intimin β protein that is essential for A/E lesion formation and infection of mice (29). Moreover, expression of intimin α from EPEC restores the ability of DBS255 to colonize the colon of orally challenged mice (10). This model provides an opportunity to evaluate the in vivo biological functions of different intimin types in mice.

In the present study, we determined the outcome of mouse inoculation with DBS255 bacteria expressing intimin γ . Since low intimin expression was observed in DBS255 bacteria expressing intimin γ from a pUC18-cloned *eae* gene (pCVD444*), we generated a hybrid intimin γ based on pCVD438 expressing intimin α . This intimin contains the receptor-binding domain

FIG. 8. (A) Weights of the distal colons of mice. (B) Crypt lengths are shown; only the DBS255(pCVD438) strain intimin produced hyperplastic crypts. (C) Numbers of CD3 $^{+}$, CD4 $^{+}$, and CD8 $^{+}$ cells in the lamina propria. In panels A through C, all mice were given 50% ethanol followed by DBS255(pCVD438) (white bars), the DBS255 strain (black bars), or DBS255(pICC55) (hatched bars) ($n = 5$; *, $P < 0.05$, Mann-Whitney U test with Bonferroni's correction). All data are shown as the mean $\pm$ 1 standard error of the mean.

of intimin γ , presented on a cloned intimin α backbone which itself is 97% identical to intimin γ . This clone of intimin α has already been shown to mediate efficient intimin expression and to restore mouse virulence to DBS255.

Before using the recombinant *eae* gene in the *C. rodentium* mouse model, we confirmed that it encodes a biologically active intimin. This was achieved by expressing the hybrid intimin γ in CVD206(pICC55) bacteria. The hybrid intimin γ was produced in CVD206(pICC55) bacteria at a level similar to that of intimin α in CVD206(pCVD438) bacteria. CVD206(pICC55) bacteria adhered and induced A/E lesions on HEp-2 cells. Importantly, this strain colonized and induced A/E lesions on human intestinal IVOC. Following these bioassays, we expressed the hybrid intimin γ in DBS255 cells. Using live DBS255(pICC55) bacteria to infect HEp-2 cells, we showed that the strain is capable of inducing A/E lesions and hence the hybrid intimin γ is biologically functional in the *C. rodentium* background and can cooperate with the other virulence factors involved in this process. The ability of the hybrid intimin γ to bind Tir-Cr was demonstrated using gel overlays and recombinant proteins. In contrast, mice oral challenges revealed that, unlike intimin α , intimin γ could not restore mouse virulence to DBS255 bacteria. This suggests intimin γ may exhibit a host cell-encoded receptor binding specificity or affinity that differs from intimin α or intimin β and that this receptor is not expressed, at least at a sufficient level, in the mouse gut. This result is consistent with our data showing different tissue tropism between CVD206 expressing α and CVD206 expressing intimin γ , using IVOC from different regions of the human gut (26).

In previous studies, we have shown that following intracolonic inoculation of formalin-killed *C. rodentium* cells expressing either intimin β or intimin α there was an extensive, Tir-independent, infiltration of CD3⁺ CD4⁺ T cells even though no bacteria were seen in association with the mucosa. For this reason, we have examined the distal colon of mice inoculated with DBS255(pICC55) cells expressing the hybrid intimin γ for signs of T-cell infiltration. None of the mice inoculated with intimin γ -encoding bacteria showed evidence of T-cell infiltration or colonic hyperplasia. These data provide further evidence that intimin γ does not bind to the mouse gut and supports the role of a host cell intimin receptor in colonization and disease. However, intimin is unlikely to be the only factor that determines host specificity, as although *C. rodentium* expressing intimin α causes colonic hyperplasia, as does intimin α when presented to permeabilized rectum on dead EPEC E2348/69 and CVD206(pCVD438) cells, these latter strains cannot colonize mouse colon or induce A/E lesions following live oral challenge. Accordingly, it seems that, like many other virulence properties, host specificity is a multifactorial and multigenic property of *C. rodentium* and EPEC.

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E.L.H. and V.H. contributed equally to this paper.

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