Enteropathogenic Escherichia coli (EPEC) are an important cause of acute and prolonged diarrhea in the developing world with between 30 to 40% of diarrhea in the first year of life being attributed to EPEC (31).

E. coli is a clonal species, with clones identified on a combination of O and H antigens. The O antigen is part of the lipopolysaccharide present in the outer membrane of gram-negative bacteria (42, 45). E. coli serogroup O55 is the third most frequent EPEC O serogroup involved in infantile diarrhea, and O55:H6, O55:H7, and O55:H– account for 90% of the strains isolated (37). EPEC and enterohemorrhagic E. coli (EHEC) can be divided into two major groups of related clones, designated EPEC clones 1 and 2 and EHEC clones 1 and 2. EPEC clone 1 typically expresses flagellar antigen H6, whereas EPEC clone 2 expresses flagellar antigens H2 or H–. EHEC clone 1 includes the O157:H7 serogroup, whereas EHEC clone 2 contains other Shiga toxin-positive E. coli serogroups. In addition, EHEC strains belonging to clone 2 are closely related to the typical EPEC strains, whereas EHEC O157:H7 is related to the atypical EPEC O55:H7 (12).

Typical EPEC strains are classified as being positive for gene probes to eae (encoding intimin), EAF (EPEC adherence factor plasmid), and bfpA (encoding bundlin, the subunit pilin of the bundle-forming pilus) (20). The O55:H– strains are considered to have lost their flagella by mutation, since the non-motile derivatives and the motile progenitor usually have the same biochemical properties. Therefore, it has been suggested that H– strains of the O55 serogroup are derived from H6 or H7 strains (37).

EPEC and EHEC have the ability to produce attaching-and-effacing (A/E) lesions on enterocytes (30, 35, 44). The A/E lesion is characterized by localized destruction of the brush border microvilli with intimate attachment of the bacteria to the enterocyte. The area underlying the bacteria consists of polymerized actin, ezrin, talin, and myosin (14) and the actin-regulating proteins WASP (Wiskott-Aldrich syndrome family of proteins) and Arp 2/3 (actin-related proteins 2 and 3 complex) (19). This host cell-bacterium interface may be raised above the host cell surface producing a pedestal. The genes necessary and sufficient for the production of an A/E lesion by EPEC are located on a pathogenicity island termed the locus of enterocyte effacement (LEE) (28). EHEC and other A/E bacteria, such as Citrobacter rodentium, also contain the LEE (4, 27) but in EHEC the LEE is necessary but not sufficient for A/E lesion production (8). Intimin, a 94- to 97-kDa protein encoded by the eae gene within the LEE, is an adherence factor, which has been shown to play a key role in intestinal colonization (12). Intimin mediates intimate attachment to the epithelial cell by binding to another LEE-encoded protein, the translocated intimin receptor (Tir), which is delivered into the epithelial cell by a type III effector system (22). The importance of intimin in human disease is highlighted by an eae mutant being significantly attenuated in human volunteers compared to the wild-type parent strain (7) and by the presence of a high titer of serum intimin antibodies in both individuals infected with EHEC (18) and in the colostrum of mothers in Brazil where EPEC infection is endemic (25).
TABLE 1. List of strains and plasmids used during this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1-Blue</td>
<td>EαA-ve competent E. coli K-12 strain</td>
<td>Stratagen</td>
</tr>
<tr>
<td>CVD206</td>
<td>eae deletion mutant of O126:H7 strain E2348/69</td>
<td>9</td>
</tr>
<tr>
<td>ICC170</td>
<td>eae deletion mutant of O157:H7 strain 85/170</td>
<td>1, 37</td>
</tr>
<tr>
<td>G21</td>
<td>Intimin α, EAF+, Bfp+, O55:H6</td>
<td>1, 37</td>
</tr>
<tr>
<td>G30</td>
<td>Intimin α, EAF+, Bfp+, O55:H6</td>
<td>1, 37</td>
</tr>
<tr>
<td>G35</td>
<td>Intimin γ, EAF+, Bfp+, O55:H–</td>
<td>1, 37</td>
</tr>
<tr>
<td>G58</td>
<td>Intimin γ, EAF+, Bfp+, O55:H7</td>
<td>1, 37</td>
</tr>
<tr>
<td>pCVD438</td>
<td>pACYC184 encoding intimin α</td>
<td>This study</td>
</tr>
<tr>
<td>pCPG1</td>
<td>pMAL-c2 encoding intimin αO55/146</td>
<td>This study</td>
</tr>
<tr>
<td>pCPG2</td>
<td>pACYC184 encoding intimin αO55/146</td>
<td>This study</td>
</tr>
</tbody>
</table>

To date, five intimin types have been identified and designated α, β, γ, δ, and ε (1, 32). Intimin α and intimin γ are associated with different evolutionary branches of EPEC and EHEC. Intimin α is associated with EPEC branch 1 and intimin γ is specifically associated with EHEC O157:H7 and the related EPEC O55:H7 (46). Intimin exchange studies in piglets have shown that varying the intimin type can alter the site of intestinal colonization by EHEC (43). This activity of intimin was further characterized by using in vitro organ culture (IVOC). The prototype intimin-α-expressing strain O127:H6 E2348/69 produces A/E lesions on duodenum, terminal ileum, Peyer’s patches and, in a small percentage of incubations, on the colon (33). In contrast, intimin γ-expressing EHEC exhibited a restricted tropism toward the follicle-associated epithelium (FAE) of Peyer’s patches (35). When intimin γ from O157:H7 was placed in an eae mutant strain of E2348/69, adherence to the FAE predominated, thus mimicking the restricted tissue tropism of O157:H7 (35). In contrast, placing intimin α in an eae mutant strain of O157:H7 resulted in colonization of the small intestine, as well as Peyer’s patches (9). Thus, intimin is able to modulate the tissue tropism of EPEC and EHEC on human intestinal explants, producing characteristic intimin α- and γ-related phenotypes.

The receptor binding activity of intimin has been localized to the C-terminal 280 amino acids (Int280) (10). The global fold of Int280α was determined by nuclear magnetic resonance (21) and crystallography (26), which showed that it is built from three globular domains with the first two comprising β-sheet sandwiches. The third C-terminal domain (residues 183 to 280) has a topology that resembles C-type lectin domains (CTLD), a family of proteins responsible for cell surface carbohydrate recognition. Analysis of intimin-Tir complexes revealed amino acids likely to be involved in binding (3, 26) and showed these residues to be concentrated in a solvent-exposed region of the CTLD. Site-directed mutagenesis of residues within the CTLD was able to modulate the biological activity of intimin; in particular, substituting a valine at 911 with alanine in intimin α produced a derivative, which bound to intestinal mucosa in an intimin γ-like phenotype (36).

We studied the EPEC O55 serogroup, which expresses different intimin types, by using in vitro organ culture to further elucidate the mechanisms involved in tissue tropism.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Strains and plasmids were grown overnight at 37°C with shaking (250 rpm) in L broth (LB; Sigma) for DNA sequencing and site-directed mutagenesis; for infection assays, bacterial strains were grown overnight without shaking in brain heart infusion broth.

Human intestinal in vitro organ culture adhesion assay. Tissue was obtained with fully informed parental consent and local ethical committee approval by using grasp forceps during routine endoscopic (Olympus PCF pediatric endoscope) investigation of intestinal disorders. Duodenal, terminal ileal, Peyer’s patch, and transverse colonic biopsies were taken from areas showing no macroscopic abnormalities. Light microscopy subsequently showed no histological abnormality. IVOC infections were performed as described previously (15). Briefly, 25 μL of an overnight bacterial culture was inoculated onto the biopsy samples from the various regions of the gut. The IVOC medium was changed every 2 h, and the assay was terminated at 8 h. Each bacterial strain was examined on several occasions by using tissue from different patients. Samples were fixed with 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide and viewed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

DNA sequencing of intimin from O55:H6 strain G21. Genomic DNA from G21 was isolated by using the DNAeasy tissue kit (Qiagen), according to manufacturer’s instructions. The 3’ end of the eae gene encoding Int280α from O55:H6 was amplified by PCR with high-fidelity Pfu DNA polymerase (1 cycle of 94°C for 1 min, followed by 25 cycles of 94°C for 30 s, 50°C for 1 min, and 74°C for 1 min, followed by 1 cycle of 74°C for 5 min; forward primer, 5’-GGA ATICATTACGAGATATTACGATTTATTTACACAAAGCTGGC-3’; reverse primer, 5’-CGGATGCTTATTTACACAAAGCTGGC-3’). The amplified DNA and the plasmid pMAL-c2 were cut by using the restriction enzymes EcoRI and BamHI. The amplified DNA was inserted into the cut pMAL-c2 by ligation with T4 DNA ligase. pMAL-c2 was transformed into E. coli XL1-Blue cells. Ampicillin-resistant transformants were randomly selected and screened for the appropriate insert. XL1-Blue colonies containing the recombinant plasmid were grown overnight in LB, the plasmids isolated by using the plasmid miniprep kit (Qiagen) and ethanol precipitation. DNA sequencing was performed with 1 μg of plasmid DNA, and the Int280 forward and reverse primers (36).

Site-directed mutagenesis. Site-directed mutagenesis of intimin α was performed by using the QuickChange site-directed mutagenesis kit (Stratagen) according to the manufacturer’s instructions. Plasmid pCVD438 encoding intimin α (5) was used as a template. A complementary mutagenesis oligonucleotide pair incorporating a single amino acid substitution was designed. Primers were as follows: forward primer (5’-CAGCTCAAGATGGAAGTTGGTGTTG-3’) and reverse primer (5’-GGCGACACGATCCACATGTTGATGCT-3’). Mutated plasmid containing staggered nicks was generated by extension of primers annealed to opposite strands of the denatured plasmid by temperature cycling in the presence of the high-fidelity Pfu DNA polymerase. Synthesized DNA containing the desired mutation was selected from the original DNA template by incubation with the enzyme DpnI at 37°C for 1 h, which cleaves the parental DNA at dam methylated GATC sequences, leaving the unmethylated newly synthesized mutated plasmid intact. Nicks in the plasmid were repaired after transformation of 1 μL of the synthesized DNA into competent E. coli XL1-Blue cells. Chloramphenicol-resistant transformants were randomly selected and grown overnight in LB. Plasmid DNA was isolated by using the plasmid miniprep kit (Qiagen) and ethanol precipitation. Correct incorporation of the mutation was monitored by DNA sequencing by using an automated DNA sequencer. The mutated plasmids were then transformed into eae deletion mutants of EPEC strain CVD206 (5) and EHEC strain ICC170 (9).
RESULTS

IVOC tissue tropism of O55 serogroup strains G21, G30, G35, and G58. Intimin, from EPEC and EHEC, is essential for A/E lesion formation in IVOC (9, 15). All of the EPEC strains expressed intimin, as shown by Western blotting with a universal intimin antiserum (2) (data not shown). SEM of the un inoculated controls showed good preservation of mucosal architecture, the epithelium was intact and there was no evidence of excess extrusion of enterocytes or mucus. All O55 strains showed a restricted pattern of A/E lesion formation, which was limited to FAE overlying ileal Peyer’s patches (Fig. 1 and Table 2). Thus, the intimin γ-expressing strains G35 (O55:H−) and G58 (O55:H7) showed the same tissue tropism as EHEC O157:H7 (35), which expresses intimin γ. Strains expressing intimin α can produce A/E lesions on duodenum and ileum, as well as Peyer’s patches (9, 33). However, O55:H6 strain G21, which expresses intimin α, did not reproduce this phenotype, forming A/E lesions on Peyer’s patches alone. In order to investigate if this phenomenon is unique to strain G21 or common to other O55:H6 strains, a second isolate, strain G30 was tested in IVOC. Like G21, strain G30 did not adhere to duodenum, terminal ileum, or colon. These results indicate that intimin α strains may show a variable pattern of tissue tropism.

TEM was used to confirm the presence of A/E lesions on intestinal Peyer’s patch explants (Fig. 2). In all three O55 strains, intimate adherence of bacteria to the enterocyte with effacement of microvilli could be seen. The bacterium-enterocyte interface was primarily seen as a depression, with only a few pedestal-like structures observed. No bacteria were observed within enterocytes.

Sequencing of intimin α from O55:H6 strain G21. Site-directed mutagenesis within the CTLD region of intimin α has shown that the mutation of a specific residue (Val911) can lead to the production of an intimin α-expressing EPEC derivative with a restricted colonization phenotype (36). To determine whether the eae gene of G21 contained any variation in this

TABLE 2. Patient age and number of biopsies with adherent bacteria expressed as a proportion of biopsies inoculated

<table>
<thead>
<tr>
<th>Strain</th>
<th>Small intestine</th>
<th>Peyer’s patch</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>G21</td>
<td>0/4</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>G30</td>
<td>0/3</td>
<td>ND</td>
<td>0/3</td>
</tr>
<tr>
<td>G35</td>
<td>0/4</td>
<td>4/4</td>
<td>0/3</td>
</tr>
<tr>
<td>G58</td>
<td>0/7</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>CVD206</td>
<td>0/5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CVD206(pCPG2)</td>
<td>5/5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ICC170(pCPG2)</td>
<td>3/3</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* The median patient ages (in months) for each group were as follows: small intestine, 115 (range, 9 to 188); Peyer’s patch, 73 (range, 30 to 171); and colon, 119 (range, 103 to 167). ND, not done.
The carboxy-terminal region encoding Int280 was compared to that of the prototype intimin from O127:H6 strain E2348/69. This revealed a single amino acid change at position 907 (Fig. 3), i.e., a valine residue in G21 instead of an alanine residue as in E2384/69. In order to assess the influence of the presence of valine at position 907 in the intimin α of G21, site-directed mutagenesis was performed on pCVD438, which contains eae from E2348/69, generating plasmid pCPG2. The plasmid was transformed into the eae mutant E2348/69 strain CVD206 and the eae mutant O157:H7 85/170 strain ICC170. These strains, CVD206(pCPG2) and ICC170(pCPG2), were tested by using the IVOC assay.

IVOC assay of CVD206(pCPG2) and ICC170(pCPG2). CVD206(pCPG2) was tested for A/E lesion formation on duodenum by using the IVOC assay. It was important to use duodenal explants as A/E lesion formation on the proximal small intestine is a reported characteristic of intimin α strains, whereas A/E lesion formation on FAE of Peyer’s patches is synonymous with strains expressing intimin α and with strains expressing intimin γ. CVD206 showed no adhesion (Table 2) as reported earlier in qualitative form. CVD206(pCPG2) produced A/E lesions on the duodenum in a manner similar to that of E2348/69 (Table 2 and Fig. 4A). This indicates that the difference in intimin amino acid sequence between G21 and E2348/69 cannot be the determining factor in the restricted tissue tropism of G21.

The use of the EPEC derived strain CVD206 as a recipient, for pCPG2, may have been a factor in the inability of CVD206(pCPG2) to reproduce the restricted phenotype associated with G21. To test this, pCPG2 was placed in ICC170, an eae mutant strain derived from EHEC 85/170 which only produces A/E lesions on follicle-associated epithelium (9). The resultant strain, ICC170(pCPG2), produced A/E lesions on duodenum in a manner similar to that of CVD206(pCPG2) (Table 2 and Fig. 4B). Therefore, the restricted tissue tropism of O55:H6 G21 is associated with a factor distinct from intimin, which is not in the EPEC E2348/69 or the EHEC 85-170 backgrounds.

DISCUSSION

The E. coli O55 serogroup is a prominent cause of infantile diarrhea. Each EPEC serogroup is made up of clones with distinct combinations of virulence factors. In the case of the O55 serogroup this results in some strains belonging to the classic EPEC genotype and others having an atypical EPEC genotype with close links to EHEC O157:H7. The similarity between O55:H7 and O157:H7 is demonstrated by their nearly identical sequences for the H7 flagellin gene and the similarity

![Image](http://iai.asm.org/)
of their eae alleles (29, 37). The eae gene encodes the outer membrane protein intimin, which allows EPEC and EHEC to adhere to human intestinal cells and produce A/E lesions (9, 15). This protein can be divided into three globular domains. The CTLD, at the carboxy terminus of intimin, is responsible for binding of intimin to its translocated receptor, Tir. The 280-amino-acid carboxy-terminal region also allows intimin to induce microvillus-like processes on Hep-2 cells (34), acts as an immune modulator (16), and may bind a host cell receptor (12). Intimin α has been show to bind β1 integrin in vitro (11), although this process is not essential for A/E lesion formation (23). In addition, intimin γ binds nucleolin, which may act as a host-derived cell surface receptor during the initial stages of EHEC adhesion (40).

Intimin can be divided according to antigenicity, and so far five distinct subtypes have been published (1, 32). The significance of the different subtypes in pathogenesis remains to be fully elucidated. Recent studies have shown a correlation between intimin type and tissue tropism (9, 33). The data showed that intimin α and intimin γ produce different tropism phenotypes in a manner that is Tir type independent. Using IVOC, intimin α has been shown to allow both EPEC and EHEC to produce A/E lesions on duodenum and Peyer’s patches, whereas intimin γ limits A/E lesion formation to duodenum. Thus, the presence of BFP, which has been considered to be an initial adhesion for EPEC colonization (6), is not synonymous with villous adhesion. Indeed, in a previous study with IVOC, BFP was suggested to be involved in three-dimensional colony formation and not initial adherence (15). Such a role would remain compatible with adhesion restricted to the FAE, i.e., to establish colony units within the gut environment, allowing nonintimate bacterium-to-bacterium adhesion and, once a quorum of bacteria have been established, to permit disaggregation and colonization of other regions of the gut.

TEM showed limited evidence of pedestals (19, 38) being formed by the three O55 strains. Rather, the intimate adhesion between bacterium and host cell membrane resulted, generally, in slight depresions in the surface of the enterocyte. A similar appearance was shown in newborn pigs infected with...
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