The eae Gene of Citrobacter freundii Biotype 4280 Is Necessary for Colonization in Transmissible Murine Colonic Hyperplasia

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Transmissible murine colonic hyperplasia is characterized by proliferation of anchored stem cells in the mucosa of the descending colon of laboratory mice and is caused by Citrobacter freundii biotype 4280. This bacterium produces attaching and effacing lesions in the descending colon prior to the onset of gross hyperplasia. By mutational analysis, the chromosomal eae gene of C. freundii biotype 4280 was shown to be necessary for colonic colonization. Conversely, bacteria cured of a 65-kb plasmid, which was identified in C. freundii biotype 4280, were not attenuated for colonic colonization or for the induction of colonic hyperplasia.

Citrobacter freundii biotype 4280 is the causative agent of transmissible murine colonic hyperplasia (4), a disease of laboratory mice characterized by epithelial cell proliferation in the mucosa of the descending colon (5). Prior to the development of colonic hyperplasia, C. freundii biotype 4280 organisms colonize the colonic mucosa and produce attaching and effacing (AE) lesions (22, 37). AE lesions are characterized by intimate bacterial adherence to enterocytes, dissolution of the enterocyte brush border, and cytoskeletal rearrangements in the cytoplasm of the enterocyte underlying the site of bacterial attachment (30). The formation of AE lesions is also a feature of enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic E. coli (EHEC) infection. EPEC includes those strains of particular serotypes that produce AE lesions and cause diarrhea in human infants (34) as well as strains that produce AE lesions and diarrhea in young rabbits (10). The role of AE lesion formation in disease is not completely understood, but these histopathologic changes are thought to contribute to EPEC pathogenesis. The EPEC strains that are pathogenic for humans and the strains that are pathogenic for rabbits can be distinguished by the high-molecular-weight plasmids that they contain. While these plasmids do not share sequence homology by Southern blot analysis, the high-molecular-weight plasmids of both human EPEC isolates and the rabbit EPEC strain RDEC-1 encode adherence factors. Human EPEC isolates have related EAF (EPEC adherence factor) plasmids of approximately 90 kb that are required for full virulence in human volunteer studies (25) and mediate localized adherence to cultured cells in vitro (2). It now appears that the bfp gene, which is present on EAF plasmids and which encodes bundle-forming pili, is responsible for localized adherence (15, 17, 38). RDEC-1 also has a virulence plasmid, with a size of approximately 130 kb (12). The plasmid-encoded RDEC-1 adhesin is the AF/R1 pilus, which mediates adherence to rabbit ileal brush borders in vitro (12). On the basis of in vitro adherence data, it has been suggested that the high-molecular-weight plasmids of the human and rabbit EPEC strains contribute to species specificity (19).

EHEC strains can be distinguished from EPEC strains in that they express Shiga-toxin and cause hemorrhagic colitis and hemolytic uremic syndrome in humans (24). EHEC strains also possess high-molecular-weight plasmids, which appear to be unrelated to the EAF plasmids (26). While its role in adherence is controversial, an EHEC plasmid has also been reported to encode a pilus that promotes attachment to cultured cells in vitro (23).

When cured of their virulence plasmids, EPEC and EHEC strains retain the ability to form AE lesions (40). Indeed, cured strains of human and rabbit EPEC, while attenuated in virulence, still produce diarrhea in humans volunteers (25) and in rabbits (9), respectively. A chromosomal locus of a human EPEC strain that is necessary for the formation of AE lesions in vitro has been identified (21). Designated eae (E. coli attaching and effacing), this gene encodes a 94-kDa outer membrane protein that appears to be but one of several loci required for the formation of AE lesions (16, 20). Nucleotide sequence homology with eae has been detected in other human EPEC strains (21), RDEC-1 (21), other rabbit EPEC strains (33), EHEC (21), some Hafnia alvei strains (1), and C. freundii biotype 4280 (37). Until now, the presence of a virulence plasmid in C. freundii biotype 4280 and the role of eae in transmissible murine colonic hyperplasia have not been established. To begin to define the bacterial factors that induce colonic epithelial proliferation in this disease, we have constructed an eae mutant of C. freundii biotype 4280. We have also cured the strain of its high-molecular-weight plasmid. Both the eae mutant and the plasmid-cured strain were orally inoculated into mice and examined for their ability to colonize and to produce disease.

MATERIALS AND METHODS

Animals. Two- to 3-week-old mice, either C3H/HeJ (Radiation Biology, Stanford University School of Medicine, Stanford, Calif.) or outbred Swiss Webster (Simonsen Laboratories, Gilroy, Calif.), were orally inoculated with 10 μl of an overnight culture of bacteria (ca. 5 × 107 CFU) or mock infected with 10 μl of sterile broth. At various time points, the animals were sacrificed and necropsied. The entire colon

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TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description and relevant phenotype</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>C. freundii</strong></td>
<td>Prototype transmissible murine colonic hyperplasia isolate</td>
<td>4</td>
</tr>
<tr>
<td>Biotype 4280</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBS120</td>
<td>Biotype 4280 with a Tn5 in the 65-kb cryptic plasmid; Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>DBS231</td>
<td>DBS120 cured of the 65-kb cryptic plasmid; Kan&quot;</td>
<td>This study</td>
</tr>
<tr>
<td>DBS255</td>
<td>Biotype 4280 with a marked deletion of the eae gene; Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>DBS280</td>
<td>DBS255 with the wild-type allele of the eae gene; Kan&quot;</td>
<td>This study</td>
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<tr>
<td><strong>E. coli</strong></td>
<td>F- hsdSB20 recA1 leuB ara proA lacY galK rpsL xyl mtl supE44</td>
<td>8</td>
</tr>
<tr>
<td>HB101</td>
<td></td>
<td>BRL</td>
</tr>
<tr>
<td>DSSa</td>
<td>F- q80lacZΔΔ recA1 hsdR30 supE44 thi- gyrA supF Δ(lacZΔM15 argF)</td>
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<tr>
<td>SY327pir</td>
<td>Δ(lac pro) argE gyrA rifC recA1 pirRK6</td>
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</tr>
<tr>
<td>SM10pir</td>
<td>thi thr leu tonA lacY supE44 recA1::RP4-2-Tc::Mu Km pirRK6</td>
<td>29</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<td>pDBS1</td>
<td>65-kb cryptic plasmid of C. freundii biotype 4280 with Tn5; Kan'</td>
<td>This study</td>
</tr>
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<td>pDBS3</td>
<td>Subclone of C. freundii biotype 4280 eae gene in pACYC184; Cm'</td>
<td>This study</td>
</tr>
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<td>pDBS4</td>
<td>Subclone of C. freundii biotype 4280 eae gene in pBlueScript KS−; Amp'</td>
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<td>pDBS14</td>
<td>4.7-kb insert from pDBS4 adapted with EcoRI linkers in pUC18; Amp'</td>
<td>This study</td>
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<td>pDBS24</td>
<td>pDBS14 with aph Kan' cassette in the eae gene; Amp' Kan'</td>
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</tr>
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<td>pACYC184</td>
<td>p15A derivative; Cm' Tet'</td>
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</tr>
<tr>
<td>pUC18</td>
<td>M13mp18 derivative; Amp'</td>
<td>42</td>
</tr>
<tr>
<td>pFSV-1</td>
<td>pACYC184 derivative with oriR6K mobRP4; Cm' Tet'</td>
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</tr>
<tr>
<td>pFSV-14</td>
<td>4.7-kb insert from pDBS14 cloned into pFSV-1; Tet'</td>
<td>This study</td>
</tr>
<tr>
<td>pFSV-24</td>
<td>4.7-kb insert from pDBS24 cloned into pFSV-1; Tet' Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pFSV-101</td>
<td>SalI fragment containing the ori of pDBS120 in pFSV-1; Cm'</td>
<td>This study</td>
</tr>
</tbody>
</table>

of each mouse was collected aseptically, weighed, and homogenized as described previously (37). Appropriate dilutions of the tissue homogenate were plated on differential media with and without antibiotic selection to determine the number of CFU of **C. freundii** per mg of tissue. Where indicated, tissue was also collected for microscopy.

**Media, bacterial strains, and growth conditions.** Bacteria were stored in Lennox L (LB) broth (GIBCO Laboratories, Gaithersburg, Md.) with 50% glycerol at −20°C. Bacteria were grown in LB broth, on LB agar, or on MacConkey lactose agar (Difco Laboratories, Detroit, Mich.), or on minimal A agar (28). Where indicated, kanamycin was added at a final concentration of 40 μg/ml, tetracycline was added at 20 μg/ml, and chloramphenicol was added at 20 μg/ml. The strains and plasmids used in this study are listed in Table 1. Biotyping was performed with API 20E strips (Analytab Products, Plainview, N.Y.).

**Recombinant DNA techniques.** Plasmid DNA was isolated by alkaline lysis (36) or, in the case of high-molecular-weight plasmids, by Triton X-100 lysis as described previously (13). Restriction digests, agarose gel electrophoresis, and reactions with the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories, Inc. [BRL], Gaithersburg, Md.), calf intestinal alkaline phosphatase (Boehringer GmbH, Mannheim, Germany), and T4 DNA ligase (BRL) were performed as described previously (36). DNA fragments were isolated from low-melting-point agarose gels. Transformation of bacteria with plasmid DNA was performed by electroporation with a Gene Pulser and Pulse Controller (Bio-Rad Laboratories, Richmond, Calif.).

**DNA hybridization.** Intact plasmid DNA or isolated DNA fragments were labeled by nick translation with biotin-14-dATP (BioNick; BRL). Total bacterial DNA was isolated as described previously (27). Both total bacterial DNA and plasmid DNA were restriction digested, separated on agarose gels, and transferred to nylon membranes by the capillary transfer method (36). The membranes were UV cross-linked (Stratalinker; Stratagene, La Jolla, Calif.) and hybridized as described previously (36). After a 5-min wash with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate (SDS) and a 15-min wash with 2× SSC and 0.1% SDS, both at room temperature, the filters were washed for 2 h at 65°C with 0.1× SSC with 0.5% SDS. A chemiluminescent system (Photogene) using XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) was then used as recommended by the manufacturer (BRL) to detect hybridization.

**Microscopy.** (i) **Light microscopy.** Tissue was fixed in neutral-buffered formalin, processed for routine paraffin embedding, sectioned, and stained with hematoxylin and eosin. Samples were then examined by light microscopy. (ii) **Transmission electron microscopy.** Tissue was fixed in glutaraldehyde, processed as described previously (37), and embedded in an Epon resin (Poly/Bed 812; Polysciences, Warrington, Pa.). Ultrathin sections were examined on a Phillips 201c transmission electron microscope.

**Construction of plasmid-cured C. freundii biotype 4280.** To mark the **C. freundii** biotype 4280 cryptic plasmid, the conjugal, suicide plasmid pJM703.1::Tn5 (a kind gift of C. Bloch, Department of Pediatrics, University of Michigan) was used to introduce random Tn5 insertions into the **C. freundii** biotype 4280 genome. Five milliliters each of SM10pir(pJM703.1::Tn5) and **C. freundii** biotype 4280 were grown to an optical density of 0.5 at 600 nm, gently mixed, and vacuumed onto a sterile 0.2-μm-pore-size filter (Nalgene Co., Rochester, N.Y.). The filter was transferred to an LB agar plate and incubated at 37°C for 3 h. The bacteria were then resuspended in phosphate-buffered saline (PBS) and plated onto minimal medium containing kanamycin, which selected for the growth of Tn5-containing **C. freundii** clones. Plasmid DNA was isolated from the pool of exconjugants and used to transform the parental **C. freundii** biotype 4280 strain. The resulting Kan' transformants, which were expected to contain Tn5 insertions in plasmid DNA, were used...
to orally inoculate mice. After 6 days, the animals were sacrificed, the numbers of CFU of Kan\(^{+}\) C. freundii biotype 4280 in the colon were determined, and individual clones were isolated. One of these clones was designated DBS120.

HB101 was transformed with plasmid DNA from DBS120. Kan\(^{+}\) transformants were found to contain the entire C. freundii plasmid. This plasmid, which was called pDBS1, was isolated from HB101(pDBS1) and digested to completion with several restriction endonucleases. The restriction fragments were then ligated to similarly digested, phosphatase-treated pFSV-1 and used to transform DH5\(\alpha\). Plasmid DNA was isolated from one of the resulting Cam\(^{+}\) clones. This plasmid, designated pFSV-101, was used to transform DBS120. After selection for Cam\(^{+}\) clones were screened for Kan\(^{-}\). The loss of pDBS1 from DBS120 was confirmed by Southern blot analysis.

**Construction of a marked eae deletion mutant of C. freundii biotype 4280.** The 4.7-kb BamHI-HindIII fragment of DNA from pDBS4 (Fig. 1) containing the C. freundii biotype 4280 eae gene was treated with the Klenow segment of DNA polymerase I, ligated to phosphorylated EcoRI linkers (New England BioLabs, Inc., Beverly, Mass.), and digested with EcoRI. After this EcoRI fragment was isolated, it was ligated to EcoRI-digested, phosphatase-treated pUC18 and used to transform DH5\(\alpha\). This plasmid was called pDBS14 (Fig. 1). pDBS14 was digested with StuI and ClaI, treated with the Klenow segment of DNA polymerase I, ligated to phosphorylated XhoI linkers (New England BioLabs), and digested with XhoI. The linearized plasmid was isolated, ligated to a SauI fragment containing the aph (aminoglycoside 3'-phosphotransferase) gene from Tn903 (41), which encodes Kan\(^{-}\), and used to transform DH5\(\alpha\). This plasmid was called pDBS24 (Fig. 1). The EcoRI fragments from both pDBS14 and pDBS24 were isolated, ligated to EcoRI-digested pFSV-1, and used to transform SY327kipr to produce pFSV-14 and pFSV-24, respectively.

The suicide plasmids pFSV-14 and pFSV-24 were mated into C. freundii by mixing a loopful of donor and recipient bacteria, taken from overnight cultures on LB agar plates, and allowing them to incubate on a fresh LB agar plate for 3 h at 37°C. The bacteria were then resuspended in PBS and plated on minimal medium with antibiotic selection. Total bacterial DNA from individual colonies were screened by Southern blot analysis to confirm homologous recombination of the suicide plasmid into the C. freundii chromosome. Individual clones were passaged three times in LB broth and plated on LB plates. These clones were then patched onto plates containing antibiotics to identify clones that had lost the plasmid marker. Southern blot analysis was used to confirm a second recombination event and loss of the suicide plasmid sequences from these strains.

**RESULTS**

**C. freundii biotype 4280 plasmids.** C. freundii biotype 4280 was found to contain two plasmids of approximately 65 and 3 kb (Fig. 2). Plasmid DNA was digested with a variety of

![FIG. 1. Physical maps of plasmids used in construction and complementation of the eae mutation. Heavy lines represent C. freundii DNA, thin lines represent vector DNA, and the hatched box represents the aph gene from Tn903. The arrows represent the open reading frames of ORFU and the eae gene. Abbreviations: B, BamHI; N, NdeI; St, StuI; S, SauI; C, ClaI; H, HindIII; E, EcoRI.](image-url)

![FIG. 2. Plasmid content of C. freundii biotype 4280. An agarose gel stained with ethidium bromide is shown. Lanes: 1, HindIII-digested λ DNA; 2, undigested plasmid DNA from C. freundii biotype 4280; 3, undigested plasmid DNA from the human EPEC strain JPN15(pMAR7); 4, undigested plasmid DNA from the O157:H7 EHEC strain EDL932.](image-url)
restriction endonucleases to determine the approximate sizes of these plasmids (data not shown). To facilitate both transfer and curing of the 65-kb plasmid, an antibiotic resistance marker, which did not abrogate virulence, was introduced into the plasmid. To accomplish this, plasmid DNA was isolated from a pool of C. freundii biotype 4280 clones with random Tn5 insertions. The pooled plasmid DNA was used to transform the parental C. freundii biotype 4280, and selection for Kan' was applied. The resulting Kan' transformants were all expected to have Tn5 insertions in either of their two plasmids. A pool of these clones was orally inoculated into a mouse. After 6 days, the colon was harvested and homogenized, and dilutions were plated on MacConkey lactose agar containing kanamycin. Kan' C. freundii clones were recovered, and one of these clones, designated DBS120, was further characterized. DBS120 was found to have the same biotype and the same growth rate in LB broth as those of its Kan' parent (data not shown). When orally inoculated into mice, DBS120 colonized the colon and produced both AE lesions and colonic hyperplasia that were indistinguishable from those produced by the parent. Southern blot analysis demonstrated that a single Tn5 was present in strain DBS120, and it mapped to the 65-kb plasmid (data not shown). HB101 was transformed with plasmid DNA from DBS120, and the Kan' clones obtained were found to contain the entire 65-kb plasmid.

Curing the 65-kb plasmid from C. freundii biotype 4280. The Kan' marker of DBS120 was found to be stable, even in the absence of selection. Acridine orange (18), SDS (39), and subinhibitory levels of both nalidixic acid (14) and ciprofloxacin (32), which have all been used to cure plasmids, did not result in curing of pDBS1 (data not shown). A genetic approach was used to cure the plasmid that relies on cloning the plasmid origin of replication. Plasmid DNA was isolated from HB101(pDBS1) and separately digested to completion with SalI, CaulI, and EcoRI. The fragments from each of these digestes were ligated to the suicide plasmid pFSV-1 that had been similarly digested. Each ligation mixture was used to transform DH5a. While pFSV-1 replication is dependent on the p protein (29), and will not be maintained in DH5a, pDBS1 did replicate in laboratory K-12 strains of E. coli. Cloning of a restriction fragment containing the pDBS1 origin of replication into pFSV-1 would be expected to allow for the replication of the hybrid plasmid and for expression of the antibiotic-resistance marker of pFSV-1 in DH5a. Several stable clones were recovered after the transformation of the SalI-digested plasmids, but none were recovered after transformation of CaulI- or EcoRI-digested plasmids. The Cam' clones were found to be Kan'. Plasmid DNA, designated pFSV-101, was isolated from one of these clones. DBS120 was transformed with pFSV-101, and selection for Cam' was applied. The resulting Cam' transformants were screened, and all were found to be Kan'. Surprisingly, after three passages in LB broth without antibiotic selection, 50% of the colonies that were tested were found to be Cam' as well. Southern blot analysis confirmed that five of the six clones tested had lost all of the sequences from the 65-kb plasmid (Fig. 3). One of these clones was designated DBS231 and further characterized.

Plasmid-cured C. freundii biotype 4280 is fully virulent. The plasmid-cured derivative DBS231 and the plasmid-containing Kan' parent DBS120 were found to have the same biotype and the same growth rate in LB broth (data not shown). When the strains were inoculated into mice, there was no significant difference between the colonization by the plasmid-cured strain and that by its parent (Table 2) (P > 0.22). The plasmid-cured strain produced gross colonic hyperplasia (data not shown) and histopathologic lesions (Fig. 4) which could not be distinguished from those produced by the plasmid-containing parental strain.

Constructing a marked deletion of the eae gene. The nucleotide sequence of a 4.7-kb BamHI-HindIII fragment of DNA from C. freundii biotype 4280 has been determined, and the fragment has been shown to contain the entire open reading frame of the eae gene of C. freundii biotype 4280 (37). An internal 1.5-kb portion was deleted from this fragment and replaced with the similarly-sized Kan' cassette from Tn903 (41), such that approximately 1.5 kb of flanking C. freundii DNA remained on either side of the foreign DNA (Fig. 1). By conjugation of a suicide vector containing this construct and homologous recombination, the marked deletion was returned to the chromosome of C. freundii biotype 4280. This produced a merodiploid with chromosomal copies of

![FIG. 3. C. freundii biotype 4280 cured of the 65-kb cryptic plasmid. A Southern blot of EcoRI-digested total bacterial DNA, probed with the 65-kb cryptic plasmid of C. freundii biotype 4280 pDBS1 and isolated from HB101, is shown. Lanes contain DNA from pDBS1 (lane 1), DBS120 (lane 2), and six independent Kan' Cm' isolates cured of the 65-kb plasmid (lanes 3 to 8).](http://iai.asm.org)

### Table 2. Comparison of the colonic colonization of 18-day-old C3H/HeJ mice by C. freundii biotype 4280 with and without the 65-kb plasmid pDBS1

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Log CFU/mg</th>
<th>n</th>
<th>Log CFU/mg</th>
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<tr>
<td>Sterile broth</td>
<td>0.00 ± 0.00</td>
<td>3</td>
<td>0.00 ± 0.00</td>
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</tr>
<tr>
<td>Plasmid + (DBS120)</td>
<td>5.34 ± 1.07</td>
<td>3</td>
<td>6.93 ± 0.06</td>
<td>3</td>
</tr>
<tr>
<td>Plasmid − (DBS231)</td>
<td>6.20 ± 1.46</td>
<td>3</td>
<td>7.14 ± 0.03</td>
<td>3</td>
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</tbody>
</table>

* The inoculum was a 10-μl volume of LB broth (sterile broth), 6 × 10⁷ CFU of the plasmid-containing DBS120, or 6 × 10⁶ CFU of the plasmid-cured DBS231, given per os.

* Mean ± standard error; the lower limit of detection was 1 CFU/mg.
both the wild-type and the mutant alleles of the \textit{eae} gene. After passage in LB broth containing kanamycin, clones were tested for Tet\textsuperscript{r}. Of the 1,500 clones tested, 6 were found to be Tet\textsuperscript{r} (0.4%). These six clones were confirmed by Southern blot analysis to have undergone a second recombinational event that resulted in the loss of the wild-type allele (data not shown). One of these clones, designated DBS255, was further characterized. Both the biotype and the growth rate in LB broth of the \textit{eae} mutant DBS255 were found to be identical to those of the Kan\textsuperscript{r} parental strain (data not shown).

\textit{eae} is a colonization factor for \textit{C. freundii} biotype 4280. When the \textit{eae} mutant DBS255 was inoculated orally into mice, no Kan\textsuperscript{r} \textit{C. freundii} CFU were recovered from the mouse intestine, while the expected number of Kan\textsuperscript{r} DBS120 CFU was recovered (Table 3). To confirm that it was the \textit{eae} mutation that was responsible for the loss of colonization, attempts were made to complement the \textit{eae} mutation in \textit{trans}. The plasmid pDBS3, which is the 4.7-kb DNA fragment containing the \textit{C. freundii} \textit{eae} gene in pACYC184, was transformed and stably maintained in DBS255 when the strain was grown in vitro. However, DBS255(pDBS3) did not colonize the colon of mice after oral inoculation (data not shown). As an alternative to complementing the \textit{eae} mutation in \textit{trans}, the marked deletion in the mutant strain was replaced with the wild-type allele of \textit{eae}. The suicide plasmid pFSV-14 was conjugated into DBS255, and homologous recombination of the vector into the chromosome was confirmed by Southern blot analysis. Individual clones were then passage in LB broth without antibiotic selection and screened for Kan\textsuperscript{r}. Of the 200 clones that were screened, 1 was found to be both Kan\textsuperscript{r} and Tet\textsuperscript{r} (0.5%). This clone was designated DBS280 and was confirmed by Southern blot analysis to have the mutant allele replaced with the wild-type allele of \textit{eae} (data not shown). When orally inoculated into mice, DBS280 colonized to the same degree as the wild-type \textit{C. freundii} biotype 4280 (Table 3; \(P = 0.0529\) at 4 days postinfection and \(P = 0.5666\) at 10 days postinfection). DBS280 also produced colonic hyperplasia that was indistinguishable from that produced by the wild-type strain (Fig. 5).

**DISCUSSION**

The naturally occurring mouse pathogen \textit{C. freundii} biotype 4280 produces an unusual disease. Colonization of the descending colon by this organism induces proliferation of the anchored stem cells near the base of the colonic crypts, which leads to epithelial hyperplasia (5). The cytokinetics of this hyperplastic mucosa are similar to those in ulcerative colitis, Crohn’s disease, and familial polyposis (3). Individuals with these proliferative bowel disorders are known to

![Image of histopathologic changes following infection with \textit{C. freundii} biotype 4280 cured of the 65-kb plasmid. A transmission electron micrograph of AE lesions in the colon of 3H/HeJ mice 7 days after oral inoculation with the plasmid-cured \textit{C. freundii} biotype 4280 DBS231 is shown. Bars, 1 (A) and 0.5 (B) \mu m.](http://liai.asm.org/)
suffer an increased incidence and an early onset of colorectal cancer. The hyperplastic state induced by C. freundii biotype 4280 can also serve to reduce the latent period for the development of chemically induced neoplasia in the colon of mice (6). While it is likely that the reduced latent period for the onset of cancer in transmissible murine colonic hyperplasia is due to the increased number of proliferating enterocytes in the colon (6), the bacterial factors that are responsible for inducing epithelial cell proliferation have not been identified. Typical isolates of C. freundii cause a variety of opportunistic infections (35) but are not associated with AE lesion formation. C. freundii biotype 4280 produces AE lesions in the murine colon prior to the onset of gross hyperplasia, and the production of AE histopathology may contribute to colonic hyperplasia. To begin to identify the virulence determinants of C. freundii biotype 4280, we have constructed an eae mutant and a plasmid-cured strain and examined them in vivo.

The classes of diarrheagenic E. coli that produce AE lesions have been shown to contain high-molecular-weight plasmids (2, 12, 26). We have identified a high-molecular-weight plasmid in C. freundii biotype 4280 which is somewhat smaller than the virulence plasmids of human EPEC, RDEC-1, and EHEC. Southern blot analysis revealed no detectable homology between this 65-kb plasmid of C. freundii biotype 4280 and total bacterial DNA from other biotypes of C. freundii, a human EPEC strain, RDEC-1, or an O157:H7 EHEC strain (data not shown). When this plasmid was cured, no difference between isogenic plasmid-containing and plasmid-cured C. freundii biotype 4280 strains was observed in mouse colonization or in disease. These data suggest that the 65-kb plasmid is not involved in the pathogenesis of transmissible murine colonic hyperplasia. It may be that, under conditions that we have not yet examined, this plasmid confers an advantage to the bacterial organism. Survival outside of the host and host-to-host transmission, in particular, were not assessed in our animal system. It may also be that this plasmid is a clonal marker for the 4280 biotype of C. freundii, but until additional isolates of this biotype are examined, the significance of the plasmid remains unclear.

It has been suggested that the eae gene product is an adhesin because in the absence of the EAF plasmid, eae mutants of EPEC do not adhere to cultured cells in vitro (21). However, the primary adhesin for EPEC in vitro is encoded on the EAF plasmid and is probably the bfp gene product (15, 17, 38). eae mutants of EPEC which carry the EAF plasmid fail to exhibit the intimate adherence to cultured cells characteristic of AE lesions but are not defective for primary adherence (21). Nonetheless, the eae locus has been shown to be a virulence determinant in human volunteer studies (16). In this study, an eae mutant of C. freundii biotype 4280 failed to colonize the colonic mucosa of mice. While it is possible that the aph fragment used to inactivate the eae gene had a polar effect on genes other than eae, the presence of extensive direct repeats immediately downstream of the eae gene (37) makes the possibility of a polar effect unlikely. Likewise, the failure of the 4.7-kb fragment of C. freundii DNA to complement the eae mutation in trans might be consistent with a polar effect of the mutation but could also be due to instability of the vector in vivo or the absence of important sequences 5' of the eae gene on the plasmid construct.

The fact that eae is necessary for persistent colonic colonization raises the possibility that intimate eae-dependent adherence is the primary mechanism for mucosal attachment by C. freundii biotype 4280 in vivo. This would be in contrast to the AE lesion-forming E. coli strains and could be accounted for either by differences in the eae gene products themselves or by differences in small-bowel versus large-bowel colonization. Initial adherence factors might be important for colonization leading to AE lesion formation.
more proximally in the gut but might not be necessary for extensive colonization and AE histopathology in the descending colon. Alternatively, C. freundii biotype 4280 might possess an as-yet-unnamed initial adhesin. If so, our data would suggest that the putative C. freundii initial adhesin is not plasmid encoded and is not sufficient for persistent colonic colonization.

The analysis of additional mutants of C. freundii biotype 4280 and the expression of biotype 4280 genes in other biotypes of C. freundii will clarify the molecular basis of mucosal colonization. These studies will also establish whether C. freundii biotype 4280 possesses a novel factor that induces epithelial proliferation or whether the formation of AE lesions in the mucosa of the descending colon is sufficient to cause transmissible murine colonic hyperplasia.

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