Distribution of the *Escherichia coli* Common Pilus among Diverse Strains of Human Enterotoxigenic *E. coli*α

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The *Escherichia coli* common pilus (ECP) is produced by commensal and pathogenic *E. coli* strains. This pilus is unrelated to any of the known colonization factors (CFs) of enterotoxigenic *E. coli* (ETEC). In this study, we investigated the distribution and production of ECP among a collection of 136 human CF-positive and CF-negative ETEC strains of different geographic origins. The major pilus subunit gene, *ecpA*, was found in 109 (80%) of these strains, suggesting that it is widely distributed among ETEC strains. Phenotypic analysis of a subset of 43 strains chosen randomly showed that 58% of them produced ECP independently of the presence or absence of CFs, a percentage even higher than that of the most prevalent CFs. These data suggest an important role for ECP in the biology of ETEC, particularly in CF-negative strains, and in human infection.

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrheal disease and mortality for children living in developing countries (11). The presence of ETEC in these areas is associated with a lack of sanitation or poor sanitation and the consumption of contaminated water or food. The major virulence factors of ETEC are a heat-labile (LT) and/or a heat-stable (ST) enterotoxin and multiple adhesive pili called colonization factors (CFs) (1, 7), which are produced in the small intestine and can cause life-threatening, cholera-like watery diarrhea (7). Since the early 1970s, more than 25 different CFs have been reported in ETEC strains of diverse geographic origins, and the prevalence of these pili differs by geographic region (7, 11). Studies of the prevalence and distribution of CFs among ETEC strains worldwide have shown that the most common CFs are CFA/I and combinations of *E. coli* surface antigens CS1, CS2, and CS3 or of antigens CS4, CS5, and CS6. Approximately 50% of ETEC strains contain at least one of these CFs (7), leaving 50% of strains that do not produce any of the CFs known or characterized so far. The presence of type IV pili, which are associated with host colonization and virulence in many gram-negative bacteria, has also been demonstrated in a significant number (30 to 50%, depending on the geographic source) of ETEC strains, including strains that do not harbor any of the known CFs. These pili provide a mechanism for the organisms to colonize the human gut and establish gastrointestinal disease. Epidemiological studies have shown that protective immunity, attributed to the antigenic variety of the CFs produced, can be achieved through multiple infections. Thus, it is believed that vaccines aimed at preventing ETEC infections, particularly in the young population and travelers, should contain the immunogenic B subunit of the LT and a combination of the most common CFs (7, 9, 10).

Previously, it was reported that meningitis-associated *E. coli* strains, and not other *E. coli* pathogroups, were able to assemble a “meningitis-associated temperature-dependent pilus” (Mat) after growth at 20°C in Luria-Bertani (LB) medium. The major pilus subunit of the Mat pilus is encoded by the *yagZ* gene, commonly found in all *E. coli* strains. Recently, our laboratory reported that most (75%) strains of human and animal *E. coli* pathogroups (including ETEC), as well as commensal *E. coli* strains, produce at 37°C a pilus adhesive structure composed of a major 21-kDa protein pilin subunit corresponding to the product of the *yagZ* gene (8). Because this gene was demonstrated to be widely distributed and highly conserved among *E. coli* strains, and because production of the pilus was shown in the major *E. coli* pathogens, it was proposed that the pilus be renamed “*E. coli* common pilus,” or ECP, and that the gene encoding the pilin subunit be designated *ecpA*. A role for ECP in adherence to cultured human epithelial cells was demonstrated in enterohemorrhagic *E. coli* (EHEC) O157:H7 and commensal *E. coli* strains (8).

ECP is not related to any of the known ETEC CFs. The present study was carried out to further investigate the presence of *ecpA* and to determine the production of ECP in a collection of human ETEC strains that had previously been characterized as CF positive or CF negative. We found ECP production in both groups of strains at rates comparable to those found for the most common CFs. Our data suggest that the production of ECP in ETEC strains may contribute to the adhesive properties of this organism and may represent a target for vaccine development and the prevention of ETEC infections.

**MATERIALS AND METHODS**

**Bacterial strains and conditions.** A collection of 136 ETEC strains from various countries, including Bangladesh, Brazil, Egypt, and Mexico, was used in this study. The collection included reference strains and clinical isolates with or without known CFs. To determine optimal conditions for ECP production, we

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selected strain EC17, a CF-negative clinical isolate from Bangladesh. It was cultured in pleuropneumonia-like organism (PPLO) broth (BD Difco, NJ) and colonization factor antigen (CFA) agar at room temperature (26°C), at 37°C with aeration, or at 37°C under a 5% CO₂ atmosphere. All other ETEC strains were grown in PPLO medium at 37°C under 5% CO₂ in subsequent experiments. EHEC O157:H7 strain EDL933 and its derivative ΔecpA mutant were used as positive and negative controls for ECP production, respectively, and were grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) at 37°C in the presence of 5% CO₂.

**Gene amplification.** ecpD in ETEC strains was amplified by PCR using GoTaq Green master mix (Promega, Madison, WI) containing 1 mM each primer. Forward primer G270 (5'-TGAAAAAAAAGTTCTGGCAATAGC-3') and reverse primer G271 (5'-CGCTGATGAGGAAATGTGAA-3') were used for the initial PCR. Primers G268 (GTCAGTCTGCACCGATGACAAAGTATCCAC), the forward primer for ecpB, and G273 (TCACCGGTAAGAATTTACCC), the reverse primer for ecpB, were used to search for a possibly modified form of ecpD in PCR-negative strains. The reaction was run through 35 cycles with a melting temperature of 94°C, an annealing temperature of 62°C, and an elongation temperature of 72°C.

**Gene sequencing.** PCR samples were prepared as described above. Samples were prepared for sequencing of the PCR-amplified ecpD gene using forward primer G489 (5'-ATGACGGCTCAGGGAATGCTGCCTAA-3') and reverse primer G490 (5'-CTGTCAGTCCTAATGATGATATCCCTAA-3') and reverse primer G490 (5'-CTGTCAGTCCTAATGATGATATCCCTAA-3'). The amplified PCR product was sequenced at Arizona Research Laboratories Division of Biotechnology, University of Arizona. Chromas, version 1.45, was used to analyze the sequence. ClustalW was used to align and compare the sequences.

**Detection of EcpA expression.** Western blotting was used to detect EcpA production by using a 1,500,000 dilution of rabbit anti-ECP antibodies and a 1:20,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) as previously described (12). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whole-cell bacterial extracts were obtained by boiling for 5 min in acidic water (pH 1.8). Sample denaturing buffer (4×) was added, and the pH was neutralized with NaOH, followed by electrophoresis in 16% polyacrylamide gels.

**Flow cytometry.** To detect the production of ECP under the various growth conditions employed, samples were processed for flow cytometry as previously described (8). Briefly, overnight bacterial cultures were incubated with anti-ECP antibodies diluted 1:1,000 in phosphate-buffered saline (PBS), followed by goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA) diluted 1:500 in PBS. Samples were analyzed in a Becton Dickinson FACScan flow cytometer.

**Transmission electron microscopy and immunogold labeling.** ECP was visualized on bacterial cultures after negative staining with 1% phosphotungstic acid (pH 7.2) using a Philips CM12 transmission electron microscope as previously described (8). Immunoelectron gold labeling was performed using rabbit anti-ECP antibodies and goat anti-rabbit IgG conjugated to 10-nm-diameter gold particles, followed by negative staining as previously described (8).

**Immunofluorescence.** Ten microliters of overnight bacterial cultures was incubated for 6 h with HT-29 human colonic cell monolayers at 37°C under 5% CO₂. After incubation and a wash with PBS, the cells were fixed with 2% formalin, and immunofluorescence was carried out to visualize ECP on bacteria adhering to HT-29 cells. Samples were incubated for 5 min at room temperature with 0.1% Triton X-100 in PBS, followed by a 30-min incubation with RNase diluted 1:5,000 in PBS with normal horse serum (PBS-HS) at 37°C. Cells were then incubated with a rabbit anti-ECP antibody diluted 1:5,000 in PBS-HS, followed by goat anti-rabbit IgG conjugated with Alexa Fluor 488 diluted 1:5,000 in PBS-HS, for 1 h at ambient temperature. A 1:5,000 dilution of propidium iodide in PBS-HS was used for DNA staining. Cells were washed with PBS after each step. Coverslips were mounted on glass slides with 1% p-phenylenediamine mounting medium. Samples were visualized under a Zeiss Axiosmager A1 microscope and photographed with a QImaging Retiga 2000R Fast 1394 camera.

**RESULTS**

High prevalence of ecpD among ETEC strains. We studied a collection of 136 human ETEC strains for the presence of the ecpA gene. These strains were clinical isolates from countries around the world, including Bangladesh, Egypt, Mexico, and Brazil. A PCR-based survey was performed using primers specific for ecpA. One hundred three (76%) strains were positive for the presence of the gene. Since it is possible that the ecpA-negative strains lacked nucleotides or contained nucleotide differences in the ecpA gene, a second PCR, using primers specific to the ecpA-flanking genes ecpR and ecpB, was performed on the PCR-negative strains, resulting in six more ecpA-positive strains. The ecpA genes from two strains were sequenced (GenBank accession numbers FJ210911 and FJ210912) and aligned using ClustalW to identify such nucleotide differences. There were 2-bp mismatches in the 3’ end of the gene, which included the reverse-primer region, compared to the ecpA sequence of ETEC strain B7A. This may account for the failure to amplify these strains. Thus, overall, 109 (80%) of the strains tested contain the ecpA gene, suggesting that it is a widespread fimbrial gene and that genetic variation exists among ETEC strains.

Growth conditions for ECP production in ETEC. Conventionally, production of most of the ETEC CFs has been demonstrated after growth on CFA agar at 37°C (1). The longus type IV pilus (also called CS21) is produced by ETEC strains grown on blood agar plates at 37°C and is not produced on CFA agar (2). In meningitis-associated E. coli strains, ECP was produced only in LB medium at 20°C (6). On the other hand, we previously demonstrated that EHEC O157:H7 produces ECP in tissue culture medium (DMEM) at either 26°C or 37°C, but not in Luria-Bertani medium. The presence of 5%
CO₂ was a stimulating factor for ECP production at both temperatures (8). Since ECP has not been observed on ETEC strains before, perhaps because this pilus is not favorably produced on CFA agar at 37°C (Fig. 1), we aimed here to study the production of ECP in ETEC under a variety of growth conditions. With this aim, we chose ETEC strain EC17, a clinical isolate from Bangladesh that does not produce any of the known CFs but produces ECP in DMEM at 37°C, as demonstrated by Western blotting (data not shown). ETEC EC17 was grown in PPLO broth and on CFA agar at 37°C under 5% CO₂, at 37°C with shaking, or statically at 26°C. We found that growth in PPLO broth at 37°C under 5% CO₂, as well as in PPLO broth and on CFA agar at ambient temperature, yielded the highest production of ECP, as determined by flow cytometry using an anti-ECP antibody (data not shown). For ease of handling a large number of strains in phenotypic experiments, we used PPLO broth at 37°C under 5% CO₂ to test the production of ECP among our collection of ETEC strains possessing or lacking known CFs.

Synthesis of EcpA. A subset of 43 randomly selected ecpA-positive ETEC strains was screened for the production of EcpA by Western blotting. Twenty-five of these 43 strains (58%) were positive for the EcpA protein. Figure 2 is a representative blot showing EcpA synthesis in several ETEC strains. Using immunoelectron gold labeling, we also visualized ECP production on five strains shown to produce EcpA by Western blotting. Prototypic strain EC17 is shown as a representative in Fig. 1. To determine if ETEC produces ECP while adhering to cultured epithelial cells, we selected 5 EcpA⁺ strains and studied them by an immunofluorescence assay (Fig. 3). These strains produced ECP upon contact with epithelial cells, suggesting that these pili potentially mediate bacterial adherence and contribute to the overall adhesive properties of ETEC.

Association of ECP and CFs. We wanted to investigate whether the production of ECP was associated with strains producing or not producing the known CFs. Our ETEC collection for this study consisted of 43 strains, of which 23 were CF negative, 11 were CF positive, and 9 were unspecified for known CFs. Six (26%) of the 23 CF-negative strains were positive for ECP, whereas 10 (91%) of the 11 CF-positive strains were also positive for ECP (Table 1). All nine remaining ETEC strains, for which no information on CF production was available, produced ECP. We also wanted to look at the distribution of ECP among strains producing different toxin types. We found that ECP production was evenly distributed among strains producing either LT, ST, or both (data not shown). In all, our data indicate that regardless of the production of CFs or enterotoxins, ECP production is a common property of this pathogroup, and thus, its expression may represent a biological advantage for host colonization, dissemination, and survival.

DISCUSSION

Diarrheal diseases lead to approximately 20% of all childhood deaths and extensive associated morbidity in developing countries, with ETEC as a leading factor (3, 4). The first step to an ETEC infection is the colonization of the small intestine, mediated by multiple CFs. More than 25 CFs have been discovered, and their frequency depends on the geographic origin of the strains (7). However, most epidemiological studies have revealed that half of the strains studied do not possess any of the known CFs. This is a clear indication that these CF-negative strains possess other intestinal adherence mechanisms. Identifying new intestinal CFs in this subset of ETEC strains is central to the design of pilus-based vaccines designed to protect against ETEC infections. A new pilus structure called ECP has been described in commensal E. coli strains and several E. coli pathogroups, including ETEC; however, the role that these pili play in human gut colonization by diarrheagenic E. coli strains remains unknown. ECP has been shown to be important for the adherence of E. coli O157:H7 and normal flora E. coli to cells in vitro (8).
In this study, we wanted to investigate how widely *ecpA* is distributed among ETEC strains and to monitor ECP production. The results of this study show that a high percentage (80%) of ETEC strains carry the *ecpA* gene, suggesting that its presence may represent a biological or ecological advantage for this organism. We also found that about 58% of the strains tested, including CF-producing and non-CF-producing strains, produced ECP. The percentage of strains that produced ECP is comparably higher than that reported for the most common CFs, such as CS3, CS6, and CFA/I, which is also an indication of the significance of ECP in cell adherence and host colonization. Importantly, we found ECP on bacteria adhering to cultured epithelial cells at 37°C under a 5% CO₂ atmosphere, indicating that the pili could be produced in vivo as well and could serve as adherence factors, especially for strains lacking the known CFs.

Most CFs are produced after the growth of ETEC strains on CFA agar at 37°C, while longus (CS21) is produced by a significant number of strains growing on blood agar plates (2,11). We found that ECP is produced by most ETEC strains tested on PPLO agar and is not produced on CFA agar. It is clear that nutritional and environmental cues are controlling the expression of the *ecp* genes. The inability of ETEC strains to produce ECP on CFA agar may be the reason why ECP has had evaded identification in ETEC in the past. The finding that 58% of ETEC strains produce ECP will make ETEC researchers aware of the need to utilize bacteriological media other than CFA agar in order to look for new CFs, particularly among CF-negative strains.

It is thought that a pilus-based vaccine against ETEC infections should contain the most common CFs (5). However, this proposed vaccine will protect only against strains producing these common CFs and will leave vaccines unprotected against a significant number of endemic CF-negative ETEC strains. ECP production might represent a very common phenotypic trait among ETEC strains, and given that these pili were demonstrated during the adherence of ETEC strains to epithelial cells in vitro, we propose that ECP plays a role as an accessory CF in CF-positive strains and as an important adherence factor in CF-negative strains. Ongoing studies aim at elucidating the role of ECP as a CF of ETEC.

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**REFERENCES**


**TABLE 1. Association of ECP with CF-positive and CF-negative strains**

<table>
<thead>
<tr>
<th>Strain type (n)</th>
<th>No. of strains positive for:</th>
<th>% ECP positive&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>ecp&lt;sup&gt;A&lt;/sup&gt;</td>
<td>ECP&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>6</td>
</tr>
<tr>
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<td>9</td>
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<tr>
<td>Total (43)</td>
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<td>29</td>
</tr>
</tbody>
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<sup>a</sup> Based on PCR results.

<sup>b</sup> As determined by Western blotting.

<sup>c</sup> Based on the number of *ecpA<sup>+</sup>* strains.