Evidence for specificity in type 4 pilus biogenesis by enteropathogenic *Escherichia coli*

Barry P. McNamara and Michael S. Donnenberg

Author for correspondence: Michael S. Donnenberg. Tel: +1 410 706 7560. Fax: +1 410 706 8700. e-mail: mdonnenb@umaryland.edu

Division of Infectious Diseases, Department of Medicine, University of Maryland School of Medicine, 10 South Pine Street, Room 900, Baltimore, MD 21201, USA

Type 4 fimbriae (pili) are surface appendages that are expressed by many species of Gram-negative bacteria. Previous studies have demonstrated that *Pseudomonas aeruginosa* can express and assemble pilin subunits from several unrelated species, indicating a common mechanism for biogenesis of type 4 pili whereby structural subunits from one system may be interchanged with those of another. In this study, an isogenic mutant of enteropathogenic *Escherichia coli* (EPEC) was constructed containing the entire tcpA gene from *Vibrio cholerae* O395, which encodes the major structural subunit of the toxin-coregulated pilus (TCP), in place of bfpA, which encodes the major structural subunit of the bundle-forming pilus (BFP). Surprisingly, expression of type 4 pilin structures and the associated phenotype of bacterial autoaggregation in culture media were not observed for cells of the EPEC strain containing tcpA nor for those containing an additional mutation in bfpF, which otherwise is associated with a hyperfimbriate phenotype. In addition, cells of a bfpA mutant EPEC strain containing plasmids designed to express either of two different chimeric type 4 pilin subunits containing segments of BfpA and TcpA also failed to form bacterial aggregates and express type 4 pilin structures. Collectively, these results indicate that the type 4 pilin assembly system of EPEC exhibits specificity with regard to pilin subunit recognition and assembly.

**Keywords:** fimbriae, bundle-forming pilus (BFP), enteropathogenic *E. coli*, toxin-coregulated pilus (TCP), type 4 pili

**INTRODUCTION**

Type 4 fimbriae (pili) are flexible surface structures that are produced by a wide range of bacterial pathogens. These pili participate in a variety of bacterial processes that include bacterial adherence to host cells, target cell specificity, twitching motility, bacteriophage adsorption and DNA uptake. In certain cases, they have been shown to play a major role in mediating colonization of mammalian hosts by pathogenic bacteria such as *Pseudomonas aeruginosa*, *Dichelobacter nodosus*, *Vibrio cholerae* and enteropathogenic *Escherichia coli* (EPEC) (Herrington et al., 1988; Voss et al., 1996; Bieber et al., 1998).

Type 4 pili appear to be largely composed of a single structural subunit that has a number of distinctive features (Strom & Lory, 1993). These features include a short positively charged leader sequence that is removed by a specific prepilin peptidase prior to filament assembly, an N-methyl modification of the first amino acid residue of the mature polypeptide and a distinctive hydrophobic N-terminus of about 30 amino acids containing an invariant glutamic acid residue at the fifth position of the mature polypeptide. Conservation of the extremely hydrophobic N-terminal region of type 4 pilins is consistent with the hypothesis that this domain participates in subunit–subunit interactions, facilitating the assembly of individual pilin subunits into a mature filament (Parge et al., 1995). In addition, the C-terminal immunogenic region is predicted to form an intrachain disulfide bond between two conserved cysteine residues that confers protein stability (Sun et al., 1997; Zhang & Donnenberg, 1996) and contains an epithelial-cell-binding domain (Sun et al., 1997; Hahn, 1997).

The type 4 family of pilin subunits can be divided into...
two groups based on the length of their leader sequence, the identity of the first residue of the mature protein and the genomic organization of genes encoding components required for their assembly. Group A pilins from *P. aeruginosa* (Johnson et al., 1986), *Neisseria* spp. (Potts & Saunders, 1988; Meyer et al., 1984), *Moraxella bovis* (Marrs et al., 1985) and *D. nodosus* (McKern et al., 1988) are synthesized as prepilin precursors with short (six to seven amino acids), basic leader peptides that undergo endoproteolytic cleavage between invariant glycine and phenylalanine residues. Thus, they contain an N-methylphenylalanine as the first residue of the mature protein. Genes encoding components required for group A pilin assembly reside in several different regions of the genome. In contrast, group B pilins containing toxin-coregulated pili (TCP) from *V. cholerae* (Shaw & Taylor, 1990), bundle-forming pili (BFP) from EPEC (Sohel et al., 1993; Donnenberg et al., 1992), long pili (longus) and CFA/III from enterotoxigenic *E. coli* (ETEC) (Taniguchi et al., 1995; Giron et al., 1994), and thin pili encoded by the IncI1 conjugal plasmid R64 (Kim & Komano, 1997) are synthesized as precursors with longer (13 to 30 amino acids) basic leader peptides that undergo endoproteolytic cleavage between an invariant glycine and a variable residue. Thus, the first amino acid of mature TcpA is methionine, and that of BfpA (bundlin) is leucine. N-Methyl modification of the first residue of group B pilin peptides has thus far only been determined for TcpA (Shaw & Taylor, 1990). The contiguous arrangement of genes encoding components required for group B pilus biogenesis is typified by the tcp gene cluster of *V. cholerae* (Ogieman et al., 1993) and the bfp gene cluster, which resides on the large EPEC adherence factor (EAF) plasmid harboured in EPEC (Stone et al., 1996; Sohel et al., 1993).

Studies from several laboratories indicate that the assembly machinery required for type 4 pilus biogenesis is conserved. Pilin subunits from species including *D. nodosus* (Mattick et al., 1987), *M. bovis* (Beard et al., 1990) and *N. gonorrhoeae* (Hoyne et al., 1992) can be expressed and assembled on the cell surface of *P. aeruginosa*. All these studies, until recently, were performed in a hyperfimbriate strain of *Pseudomonas* that lacked twitching motility. This strain was later found to contain a mutation in *pilT*, a gene that encodes a nucleotide-binding protein that may participate in pilus retraction (Whitchurch et al., 1991). Koomey (1995) suggested that this background could remove constraints that otherwise would interfere with specific interactions between certain components of the fimbrial assembly system and pilin subunits derived from different species. However, Watson et al. (1996) have recently shown that *P. aeruginosa* lacking *pilA* in an otherwise wild-type background can assemble subunits from *D. nodosus* into fully functional pilin, as demonstrated by restoration of both twitching motility and susceptibility to fimbrial-specific bacteriophages. Whether assembly of type 4 pilin from a heterologous host is restricted to pilin composed of group A subunits or is a particular trait of type 4 pilus biogenesis in *P. aeruginosa* is not known. Studies involving expression of type 4 pilin in heterologous species have shown utility in the design of recombinant vaccines such as those against bovine keratoconjunctivitis (Lepper et al., 1995). By extending these applications to recombinant strains containing pilin subunits from group B, vaccine candidates could be designed against pathogens that predominantly cause gastrointestinal diseases in humans.

In this study, we sought to test whether components encoded by the *bfp* gene cluster from EPEC can direct the assembly of functional type 4 pili encoded by *tcpA* from *V. cholerae* O395. To do so, we constructed a derivative of EPEC in which the first gene of the *bfp* gene cluster, *bfpA*, encoding the major structural subunit of BFP, was replaced with the *tcpA* gene encoding the major structural subunit of TCP. We also examined whether a *bfpF* mutation, like that of the homologous *pitT* mutation, would facilitate expression of type 4 pili encoded by *tcpA*, since cells of a *bfpF* mutant are hyperfimbriate compared to wild-type EPEC (Bieber et al., 1998). Finally, we tested whether chimeric pilin subunits containing segments of BfpA and TcpA could be expressed and assembled in an EPEC strain containing a mutation in *bfpA*.

**METHODS**

**Bacterial strains, plasmids and growth media.** Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used to maintain all plasmids except derivatives of the positive-selection suicide vector pCVD442, which were maintained in *E. coli* DH5α pir. *L. lactis* (LB) broth (1:1:10 g tryptone, 5 g yeast extract and 5 g NaCl) or LB agar (LB containing 15% agar) were used as culture media unless otherwise indicated. Antibiotics were added, when necessary, at the following concentrations: 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹ and 50 µg nalidixic acid ml⁻¹.

**Recombinant DNA constructions and nucleotide sequencing.** DNA restriction endonuclease digests, electrophoresis, and ligations were performed by standard procedures (Sambrook et al., 1989). Plasmids were introduced into competent cells of *E. coli* strains by heat-shock transformation or electroporation. Electroporations were carried out in 10% (v/v) glycerol in 0.1 cm cuvettes with an *E. coli* pulser (Bio-Rad) set at 1-8 kV. PCR experiments, unless otherwise stated, were performed in 100 µl volumes containing 100 ng template DNA, 0.1 µM appropriate primers, 0.2 mM deoxynucleotide triphosphates, 1 x Thermopol buffer, 4 mM MgSO₄ and 0.5 U DeepVent Polymerase (New England Biolabs) in accordance with the manufacturer’s instructions. Reactions were subjected to initial denaturation of 2 min at 94 °C followed by 29 cycles of 48 °C for 45 s, 72 °C for 2 min and 95 °C for 1 min.

To replace the full-length portion of *bfpA* with *tcpA*, pLCK1 template DNA and primers Donne-36 (5′-CCAAAAGCAT-TCGAAAGATCATC-3′) and Donne-232 (5′-AGATCT-TTTATGAGTAACATGTTAACCT-3′) which encodes a UAA stop codon (bold faced) and unique BglII and Spel linker restriction sites (italicized), were used to PCR amplify a 16 kb fragment containing downstream *bfpA*. 

720
sequences. Similarly, primers Donne-26 (5’-AACGCGGGGAGGCAAGACA-3’) and Donne-233 (5’-AGATCTAACCATAATGAACTTTTCTTTTTCTTTTTTT-3’), which encode an AUG start codon (bold faced) and unique NdeI and BglII linker restriction sites (italicized), were used to PCR amplify a 3 kb fragment containing upstream bfpA sequences. PCR products were cloned separately into pCR-Script, resulting in plasmids pBPM14 and pBPM15, respectively. To destroy the NdeI site present in bfpB, a 527 bp NdeI-XbaI fragment was excised from pBPM14. Both 3’ recessive ends were filled in using DNA polymerase I large (Klenow) fragment and were subsequently religated. A 3.3 kb NotI–BglII fragment excised from pBPM15 was cloned into similar sites of pBPM14, resulting in pBPM16. Additionally, a 1.4 kb SacI fragment was excised from pBPM16 to remove a SalI site encoded by pACYC184 sequences. Template DNA derived from V. cholerae was used.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
</tr>
</thead>
</table>
| **Strains**
| E. coli  E2348/69 | Prototype O127: H6 EPEC strain | Levine et al. (1985) |
| UMD932 | E2348/69 ΔbfpA::tcpA, bfpP::aphA-3::bfpP | This study |
| UMD936 | E2348/69 ΔbfpA::tcpA | This study |
| UMD940 | E2348/69 ΔbfpA::tcpA, bfpP::aphA-3::bfpP | This study |
| DH5α | ΔlacU169 recA1 endA1 gyrA96 relA1 | Sambrook et al. (1989) |
| DH5α ppir | DH5α(3pir) | Menard et al. (1993) |
| V. cholerae O395 Sm | Classical O1 Ogawa strain; SmR | Taylor et al. (1987) |
| BGD4 | tcpA | R. Taylor* |
| **Plasmids**
| pCR-Script | High-copy-number cloning vector, CmR | Stratagene |
| pJPN101 | EAF region II encoding bfpA–L cloned into pJJK68 | Nataro et al. (1987) |
| pLKCI | pACYC184 containing 4.2 kb HindIII fragment from pJPN101 | This study |
| pLKDEL1 | pCR-Script containing 3–4 kb PCR fragment encoding the first 123 residues of BfpA and upstream sequences | This study |
| pLKDEL2 | pCR-Script containing 1.7 PCR fragment encoding last 11 residues of BfpA and downstream bfp sequences | This study |
| pLKDEL3 | pLKDEL2 containing 2.1 kb SpeI–EcoRV fragment from pLKDEL1; encodes in-frame bfpA deletion | This study |
| pWKS30 | Low-copy-number cloning vector, AmpR | Wang & Kushner (1991) |
| pBPM14 | pCR-Script containing 1.6 kb of sequence downstream of bfpA | This study |
| pBPM15 | pCR-Script containing 3.2 kb of sequence upstream of bfpA | This study |
| pBPM16 | pBPM14 containing 3.3 kb NotI–BglII fragment from pBPM15 | This study |
| pBPM17 | pBPM16 containing 674 bp NdeI–SpeI fragment from pBPM18 | This study |
| pBPM18 | pCR-Script containing 685 bp PCR fragment encoding tcpA | This study |
| pBPM21 | pCVD442 containing tcpA flanked by 1350 bp of upstream and 1050 bp of downstream bfp nucleotide sequence | This study |
| pBPM22 | pCR-Script containing 1.5 kb PCR fragment encoding first 58 residues of BfpA and upstream sequences | This study |
| pBPM23 | pCR-Script containing 1.6 kb PCR fragment encoding last 136 residues of TcpA and downstream bfp sequences | This study |
| pBPM26 | pBPM23 containing 1.6 kb KpnI–SpeI fragment from pBPM22 | This study |
| pBPM27 | pWKS30 containing 1 kb BamHI–PstI fragment from pBPM26 | This study |
| pBPM33 | pCR-Script containing 211 bp PCR fragment encoding 67 residues of the intrachain disulfide bond region of TcpA | This study |
| pBPM34 | pLKDEL3 containing 201 bp SpeI fragment from pBPM33 | This study |
| pBPM35 | pWKS30 containing 1 kb BamHI–PstI fragment from pBPM34 | This study |
| pKDS8.2 | pCVD442 containing Asp700 fragment encoding bfpF::aphA-3 | Anantha et al. (1998) |
| pRPA100 | pWKS30 containing 1 kb BamHI–PstI fragment encoding bfpA and bfpG′ | R. Anantha, University of Maryland |

* Dept of Microbiology, Dartmouth Medical School, USA.
with primers Donne-250 (5'-GGGACTAGTTATAGTGTTACAAATGTGAGCCGCGC-3'), which encodes a UAA stop codon (bold faced) and a unique SpeI restriction site (italicized), and Donne-251 (5'-GGCCGATATCGATTTATATTAAACACGGC-3'), which encodes a unique NdeI restriction site (italicized) containing an AUG start codon (bold faced), to amplify tcpA. PCR products were cloned into pCR-Script, resulting in pBPM18. A 674 bp NdeI–SpeI fragment was excised from pBPM18 and cloned into similar sites of pBPM16. The resulting plasmid, pBPM17, contains approximately 1350 bp and 1050 bp of upstream and downstream nucleotide sequence from EPEC, respectively, flanking the complete nucleotide sequence of tcpA. Both pBPM17 and pCVD442 were digested with SalI, ligated and subsequently digested with Sall to remove pCR-Script vector sequences. The resultant plasmid, pBPM21, was used as a positive selection suicide vector.

To replace sequences encoding the leader region of TcpA with those of BfpA, primers Donne-291 (5'-TGAGTTGTTGGATCTTGCGTGTCGCTTTAGACTGTTAG-3') and Donne-294 (5'-GGGGATCCTGCATAATATTTAGCTGAGTTGTTGTTGGATCTTGCGTGTCG-3') were used to amplify a 1.5 kb fragment from pLKC1. The resultant PCR product contained upstream bfp gene cluster DNA and sequences encoding the first 58 amino acid residues of BfpA. Similarly, template DNA derived from pBPM17 was used with primers Donne-289 (5'-GGGATCTGACACAGAC-3') and Don-1 (5'-AGAATCAGTTGACACAGAC-3') to amplify on 1 kb fragment encoding the last 156 amino acid residues of TcpA and remaining downstream bfp sequences. The junction site chosen represents a region of similar primary and predicted secondary structure shared between both molecules. We also note that the inclusion of SpeI sites in primers Donne-289 and Donne-291 (italicized) does not change the amino acid sequence of either pilin molecule. PCR products were cloned separately into pCR-Script to create pBPM22 and pBPM23, respectively. A 1.6 kb KpnI–SpeI fragment was excised from pBPM22 and cloned into similar sites of pBPM23 to produce pBPM26. A 1 kb BamHI–PstI fragment was subsequently excised from pBPM26 and cloned into similar sites of the low-copy-number vector pWK530 to make plasmid pBPM27.

To replace bfpA sequences that encode a region inclusive of the intrachain disulfide bond of bundlin with homologous sequences derived from tcpA, PCR amplification was first used to construct pLKCDEL3, a plasmid that encodes an in-frame deletion of codons 123 through 183 in bundlin. Briefly, pLKC1 and primers Donne-21 and Donne-26 (5'-GGCACTAGTTAAGGATCTGACCTAGTTAACAAA-3'), which contains a unique SpeI site (italicized), were used to amplify a 3.4 kb fragment containing sequences upstream of bfpA and those encoding the first 122 amino acid residues of bundlin. Similarly, primers Donne-31 and Donne-27 (5'-GGCACTAGTTAAGGATCTGACCTAGTTAACAAA-3'), which contains a SpeI site (italicized), were used to amplify a 1.7 kb fragment containing downstream bfp gene cluster sequences including those that encode the last 10 amino acid residues of bundlin. Both fragments were cloned separately into pCR-Script to produce plasmids pLKCDEL1 and pLKCDEL2, respectively. A 2.1 kb SpeI–EcoRV fragment was excised from pLKCDEL1 and cloned into corresponding sites of pLKCDEL2 to create pLKCDEL3. Primers Donne-32 (5'-GGCACTAGTTAAGGATCTGACCTAGTTAACAAA-3') and Donne-31 (5'-GGCACTAGTTAAGGATCTGACCTAGTTAACAAA-3') were used to amplify a 211 bp fragment from plasmid pBPM17 and was subsequently cloned into pCR-Script. The resultant plasmid, pBPM33, encodes amino acid residues 120 through 186 of TcpA. Two SpeI sites, each of which was designed in Donne-327 and -311 (italicized), were used to excise a 201 bp fragment from pBPM33 that was subsequently cloned into the corresponding site of pLKCDEL3 to make pBPM34. A 1 kb BamHI–PstI fragment was excised from pBPM34 and cloned into similar sites of pWKS30 to make pBPM35. Plasmid constructs were verified by restriction analysis and also by sequence analysis of both DNA strands using a PRISM Ready Reaction DyeDeoxy Termination kit (Applied Biosystems). Sequencing reactions were run on a model 373 DNA sequencer (Applied Biosystems) by staff of the University of Maryland Biopolymer Laboratory.

Molecular genetic techniques. The tcpA gene was first introduced into wild-type EPEC by mobilizing pBPM21 from DH5αpir into E2348/69 by triparental conjugation. Allelic exchange was performed as previously described (Donnenberg et al., 1993) to produce strain UMD936. To introduce a bfpF mutation into strain UMD936, plasmid pKDS82 was mobilized from DH5αpir into strain UMD936 to create strain UMD940. Candidate colonies of recombinant EPEC strains possessing mutant bfp alleles were verified by whole-cell PCR using fresh single colonies and Tagq DNA polymerase in 100 µl volumes. Reactions were subjected to initial denaturation of 2 min at 94 °C followed by 25 cycles of 45 s at 45 °C, 30 s at 94 °C for 2 min and 95 °C for 1 min. Primers Donn-5 (5'-AACACAGGGATACAAAAAGA-3') and Donne-6 (5'-TCTTGGTTCTTGCGGTGTC-3') were used to amplify sequences flanking bfpA and for sequencing the entire tcpA gene residing within the bfp gene cluster from strain UMD936. Primers Donne-235 (5'-GGGAATTCTCTGATGTTACATGATG-3') and Donne-236 (5'-GGGGATCTGACATATAATTTGAGCTAACAGTT-3') were used to amplify bfpF. Plasmid pRPA100 was electroporated into electrocompetent cells of UMD936 to complement the bfpA mutation.

Tissue culture and adherence assays. HEp-2 cells (ATCC CCL 23) in monolayer culture were used as the in vitro model for EPEC adherence studies as detailed previously (Donnenberg & Nataro, 1995). Briefly, HEp-2 cell monolayers were grown to near confluence in eight-well chamber slides (Nunc) in Eagle’s minimal essential medium with 10% fetal bovine calf serum in a 5% CO2 atmosphere at 37 °C. Adherence of EPEC cultures to HEp-2 cells was performed in the presence of 1% d-mannose. Samples were examined under brightfield microscopy without prior knowledge of their identity.

Immunoblot analysis. Induction of BFP expression from EPEC was performed by inoculating 10 ml Dulbecco’s modified Eagle’s medium/F-12 (DMEM/F-12) with 100 µl of LB-grown overnight cultures. DMEM/F-12 cultures were aerated (225 r.p.m.) at 37 °C for 4–6 h. Turbidity of each culture (measured as OD492) was normalized for immunoblot analysis. Induction of TCP expression from V. cholerae was performed as previously described (Taylor et al., 1987). Whole-cell bacterial proteins were prepared by boiling samples for 10 min in dissolving buffer (625 mM Tris/HCl, pH 68; 2%, w/v, SDS; 10%, v/v, glycerol; 5%, v/v, 2-mercaptoethanol; 0.001% bromophenol blue) and subjected to SDS-PAGE (15%, w/v, acrylamide). Resolved proteins were electrotransferred to Immobilon-P PVDF membranes using a semidysh Multiphor II NovaBlot transfer apparatus (Pharmacia) as described by the manufacturer. Membranes were blocked initially with PBS containing 01% (w/v) Tween 20 (PBST) and 5% (w/v) nonfat dry milk and then subjected to sequential 1 h incubations with either rabbit polyclonal anti-BfpA antibodies, or rabbit polyclonal anti-TcpA peptide 6 antibodies, which recognize the C-terminal portion of TcpA including the region predicted to form the intrachain disulfide.
bond (Sun et al., 1997), diluted 1:5000 in PBST, and then with goat anti-rabbit IgG antibodies (diluted 1:25000 in PBST) conjugated to alkaline phosphatase. Immunoreactive proteins were visualized using standard procedures (Harlow & Lane, 1988).

Transmission electron microscopy. BFP were visualized by growing cells of EPEC strains in DMEM/F-12 as described above. However, cultures were subjected to brief centrifugation (12000 g, 1 min) and gently resuspended in 1 ml fresh DMEM/F-12. Cultures (10 µl) were spotted onto Formvar-carbon-coated copper grids, blotted, washed three times with sterile double-deionized water and stained with 1% (w/v) phosphotungstic acid (pH 7.2). Grids were examined on a JEOL JEM-100X transmission electron microscope.

RESULTS

Construction of recombinant EPEC strains bearing full-length tcpA in place of bfpA

To determine if components encoded by the bfp gene cluster from EPEC can direct the assembly of hetero-

![Diagram](image)

**Fig. 1.** Schematic representation of the bfp gene cluster from EPEC illustrating the construction of various alleles encoding type 4 pilin subunits used in this study. (a) The entire bfp gene cluster along with upstream sequences. Arrows indicate bfp genes. Selected restriction sites used for cloning are shown. (b) The expanded view illustrates the region of the bfp gene cluster used to construct strain UMD936. The positive-selection suicide-based plasmid pBPM21, which contains a region of approximately 3 kb including tcpA sequences (denoted by the black arrow) in place of bfpA, was used for allelic exchange with the wild-type EPEC strain E2348/69. (c) Plasmid pRPA100 consists of a 1 kb BamHI–PstI fragment containing bfpA and bfpF cloned into the low-copy-number plasmid pWK530. One-kilobase BamHI–PstI fragments containing gene fusions, which encode chimeric pilin subunits possessing the first 58 amino acid residues of bundlin and the last 156 amino acid residues of TcpA, or bundlin subunits possessing amino acid residues 120 through 186 of TcpA, were cloned into similar sites of pWK530 to create plasmids pBPM27 and pBPM35, respectively. Filled areas indicate nucleotide sequences that encode corresponding regions of TcpA.

EPEC strains possessing full-length tcpA fail to autoaggregate in culture and adhere poorly to cultured HEp-2 cells

Since both BFP and TCP are required for autoaggregation of cells of EPEC and *V. cholerae* in culture, we tested whether TCP, if expressed and assembled on cells of UMD936 and UMD940, could also mediate formation of bacterial aggregates when cells were grown in culture media. Whereas cells of wild-type EPEC formed visible aggregates in culture, those of both recombinant strains UMD936 and UMD940, as well as UMD901, which lacks BFP due to a point mutation in bfpF, were incapable of autoaggregation even after extended incubation periods (data not shown). Since BFP are required for localized adherence of EPEC to host cells *in vitro*, we tested whether TCP could also mediate some level of adherence of bacteria to HEp-2 cells. As expected, cells of wild-type EPEC adhered in a pattern typical of localized adherence (Fig. 2a). Cells of both recombinant EPEC strains containing tcpA, like those of strain UMD901, adhered to HEp-2 cells in a manner not typical of the three-dimensional morphology associated with localized adherence (Fig. 2b and data not shown).

Since the presence of tcpA within the bfp gene cluster could in theory have an unanticipated adverse polar effect upon downstream gene expression, we tested whether the reintroduction of bfpA alone into cells of strain UMD936 could restore assembly of functional BFP. Cells of UMD936, when transformed with pRPA100 encoding wild-type bfpA, were capable of performing localized adherence that was indistinguishable from that of the wild-type (Fig. 2c) and formed visible bacterial aggregates when grown in culture (data not shown). As expected, the complementing plasmid pRPA100 was capable of restoring both the localized
**Fig. 2.** Localized adherence of EPEC strains to HEp-2 cells. HEp-2 cells were incubated for 3 h with bacteria of the indicated strains, fixed, stained with Giemsa and examined by brightfield microscopy. (a) Wild-type EPEC strain E2348/69. (b) EPEC strain UMD936, containing tcpA in place of bfpA. (c) EPEC strain UMD936 transformed with pRPA100.

**Fig. 3.** Expression of various alleles encoding type 4 pilin subunits from cells of *V. cholerae* O395, *V. cholerae* strain BGD4 (ΔtcpA), wild-type EPEC E2348/69, UMD901 (bfpAS129C), UMD932 (ΔbfpP::aphA-3::bfpP), UMD936 (ΔbfpA::tcpA) and UMD940 (ΔbfpA::tcpA, bfpF::aphA-3::bfpF). Samples from whole-cell lysates were adjusted for culture density, separated by 15% SDS-PAGE, transferred to PVDF, and probed with polyclonal rabbit antiserum that recognizes either the extreme C-terminal portion of TcpA including the region predicted to form the intrachain disulfide bond (a, b) or bundlin (c, d). Arrows indicate prepilin and mature pilin proteins. The positions of molecular mass markers are indicated on the right.
Specificity of type 4 pilus assembly in EPEC adherence and autoaggregation phenotypes in cells of UMD901 (data not shown). These results indicate that the assembly machinery encoded by the bfp gene cluster in UMD936 is functionally intact and is capable of assembling BFP but not heterologous type 4 pili encoded by tcpA.

Recombinant strains of EPEC fail to process TcpA completely

Prepilin subunits of the type 4 pilin family must undergo proteolytic cleavage of their signal sequence before they become available for polymerization into a helical structure. Since the biogenesis of type 4 heterologous pili from EPEC would be dependent upon cleavage of unprocessed TcpA by BfpP, the endogenous prepilin peptidase, we examined the proteolytic fate of TcpA from cells of UMD936 and UMD940 by immunoblot analysis. Unlike the single mature species of TcpA that was detected in lysates derived from cells of V. cholerae, two immunoreactive proteins of approximately 23 kDa and 20 kDa, representing unprocessed and mature forms of TcpA, respectively, were detected in lysates from both UMD936 and UMD940 (Fig. 3a). No immunoreactive product was detected from lysates of the tcpA mutant V. cholerae strain BGD4 (Fig. 3a). To determine if the tcpA gene present in the bfp gene cluster affected BfpP function, we also assessed the proteolytic fate of bundlin (the bfpA gene product) from cells of UMD936 transformed with pRPA100. As shown in Fig. 3(c), only a single lower-molecular-mass species representing mature bundlin was detected in lysates of wild-type EPEC and the UMD936 transformant. In contrast, a single higher-molecular-mass species representing pre-bundlin was only detected in the bfpP mutant strain UMD932 (Fig. 3c). These results indicate that processing of TcpA by BfpP from cells of either UMD936 or UMD940 is incomplete. They also demonstrate that failure to fully process TcpA in these strains is not due to a defect in BfpP function since cells of UMD936 were still capable of completely processing BfpA.

Recombinant strains of EPEC fail to assemble type 4 pili encoded by tcpA

Although both recombinant EPEC strains UMD936 and UMD940 failed to process TcpA completely, the amount processed could in theory represent a large enough pool of pilin subunits available for pilus assembly. In addition, the failure of cells of either strain to form autoaggregates in culture and to adhere to HEP-2 cells could indicate that TCP are simply incapable of mediating the localized adherence and autoaggregation phenotypes in EPEC. Therefore, we examined both recombinant strains for the presence of type 4 pilin structures by negative staining and transmission electron microscopy. As demonstrated in Fig. 4(a), BFP were observed from cells of wild-type EPEC. Likewise, similar structures were consistently observed from cells of the UMD936 transformant strain containing pRPA100 (Fig. 4c) but not from those of untransformed strain UMD936 (Fig. 4b). Similarly, type 4 pilin structures were only observed from transformants of UMD901 containing pRPA100 and not from untransformed strain UMD901 or from strain UMD940 (data not shown). Furthermore,
we did not detect evidence of TcpA export nor assembly of visible pili when we examined shear preparations from cells of strain UMD936 or when we examined recombinant cells by immunogold electron microscopy (data not shown). These results indicate that the available pool of processed TcpA pilin subunits fails to assemble into observable pilin structures. Thus, failure of both recombinant strains of EPEC to adhere to HEp-2 cells \textit{in vitro} and to form autoaggregates in culture is associated with the inability to express heterologous type 4 pili.

Expression of chimeric type 4 pilin subunits from EPEC is not sufficient to reconstitute type 4 pilus biogenesis

Since recombinant strains of EPEC fail to assemble TcpA subunits into a mature filament, we sought to determine if chimeric type 4 pilin subunits could be expressed and assemble in EPEC. To do so, we constructed plasmid pBPM27 to encode chimeric pilin subunits containing the first 58 amino acid residues of BfpA and the last 156 amino acid residues of TcpA (Fig. 1). Similarly, we constructed plasmid pBPM35 to encode chimeric bundlein subunits containing a region inclusive of the intrachain disulfide bond of TcpA in order to assess whether substitution of this region would be permissive for subsequent pilin subunit recognition and filament assembly (Fig. 1). Both plasmids were constructed to be identical to the complementing plasmid pRPA100, which contains \textit{bfpA} and upstream promoter sequences, except for those regions that encode specific portions of TcpA in place of bundlein. Both transformant strains of UMD901, containing either pBPM27 or pBPM35, were examined for their ability to adhere to HEp-2 cells, autoaggregate, process chimeric type 4 prepilin subunits and assemble type 4 pili. Cells of either transformant adhered poorly to HEp-2 cells in a manner that was indistinguishable from that of the parent strain UMD901 (data not shown). Interestingly, neither processed nor unprocessed chimeric type 4 pilin subunits were detected in whole-cell lysates of either UMD901 transformant regardless of whether anti-TcpA (Fig. 3b) or anti-bundlein (Fig. 3d) antibodies were used. As expected, processed bundlein was detected by antibuclidean antibodies from lysates of strain UMD901 transformed with pRPA100 and from wild-type EPEC (Fig. 3d). Thus, it appears that the inability of either UMD901 transformant to carry out functions mediated by type 4 pili is due to the instability of both type 4 pilin chimeric subunits.

**DISCUSSION**

Type 4 pili are expressed from a wide range of Gram-negative bacteria. Despite the diversity of species that possess these structures, it appears that type 4 pili represent a cohesive group that are structurally and functionally related. Conservation of the arrangement and identities of homologous genes involved in pilus assembly from various species indicates that pilin subunits are assembled by a common mechanism and suggests that they may be interchanged among compatible hosts. This is exemplified by the work of several groups who have demonstrated that \textit{P. aeruginosa} can assemble fimbrial subunits of \textit{D. nodosus}, \textit{M. boudi} and \textit{N. gonorrhoeae} into mature filaments on the cell surface (Watson \textit{et al.}, 1996; Hoyne \textit{et al.}, 1992; Beard \textit{et al.}, 1990; Mattick \textit{et al.}, 1987). Despite the ability of these groups to interchange various group A pilin subunits, our attempts to substitute one group B pilin subunit, BfpA from EPEC, with another, TcpA from \textit{V. cholerae}, were unsuccessful. Cells of UMD936, the EPEC recombinant strain containing the full replacement of \textit{bfpA} with \textit{tcpA}, consistently failed to assemble type 4 pili as judged by their failure to autoaggregate in culture, adhere to HEp-2 cells \textit{in vitro}, and assemble any type 4 pilin structures. Two observations suggest that this failure was not due to the manner in which the \textit{bfpA} gene was replaced with \textit{tcpA} \textit{in situ}. First, this replacement was precise, as no other sequences within the \textit{bfp} gene cluster were altered other than those which encoded bundlein. Second, reintroduction of \textit{bfpA}, encoded on pRPA100, into cells of UMD936 resulted in their ability to perform localized adherence, autoaggregate and assemble processed bundlein into mature filaments. Thus, we conclude that the assembly machinery encoded by the \textit{bfp} gene cluster in UMD936 is functionally intact, but is incapable of assembling type 4 pilin composed of TcpA. We also examined whether a \textit{bfpF} mutation would facilitate expression of type 4 pili encoded by \textit{tcpA} from EPEC since cells of a \textit{bfpF} mutant, like those of a \textit{pilT} mutant, produce many more bacterial filaments than do wild-type cells (Bieber \textit{et al.}, 1998; Bradley, 1974). Similar to our earlier results, cells of strain UMD940 containing \textit{bfpF} and the \textit{tcpA} substitution also failed to autoaggregate in culture, adhere to HEp-2 cells \textit{in vitro}, and assemble any detectable surface structures resembling type 4 pili. Thus, failure to detect any type 4 pilin structures encoded by \textit{tcpA} from cells of UMD936 would not necessarily be the result of our limits of detection.

EPEC and \textit{V. cholerae} O395 are both enteric pathogens that express group B type 4 pili. Conservation of the type 4 pilin assembly genes of these organisms suggested that EPEC would be capable of expressing type 4 pili encoded by \textit{tcpA} from \textit{V. cholerae}. Genes required for TCP or BFP biogenesis appear to be organized or clustered in a single operon (Ramer \textit{et al.}, 1996; Brown & Taylor, 1995). Both gene clusters begin with the gene encoding the major structural subunit, \textit{tcpA} and \textit{bfpA}, respectively; these two genes display 30.4\% identity. These genes are followed by a region of dyad symmetry that is predicted to regulate expression of downstream loci and the clusters conclude with the remaining 13 genes, many of which encode proteins that share sequence similarities (Ogiierman \textit{et al.}, 1993; Sohel \textit{et al.}, 1996; Stone \textit{et al.}, 1996). These include putative nucleotide-binding proteins BfpD and TcpT; integral outer-membrane lipoproteins TcpC and BfpB; cytoplasmic-membrane-associated proteins BfpE and TcpE; and prepilin peptidases BfpP and TcpJ. BFP and TCP also share...
several functional similarities including the bundling of individual filaments and the formation of bacterial aggregates in culture (Taylor et al., 1987; Vuopio-Varkila & Schoolnik, 1991), which may facilitate colonization of the host in vivo (Bieber et al., 1998). Despite the fact that EPEC and V. cholerae share many features of type 4 pilus biogenesis, EPEC fail to assemble pili encoded by tcpA.

Our studies indicate that the prepilin peptidases of EPEC and V. cholerae may not be ideal functional homologues since EPEC fail to process TcpA completely. We note that V. cholerae possesses two distinct prepilin peptidases, TcpJ, which is required for TcpA processing (Kaufman et al., 1991), and TcpD, which is required for assembly of the type 4 mannose-sensitive haemagglutinin (MSHA) pilus and cholera toxin secretion (Marsh & Taylor, 1998). The presence of these two prepilin peptidases, which by themselves exhibit limited functional homology (Marsh & Taylor, 1998; Kaufman et al., 1991), contrasts with the reciprocal functional homology that is displayed between the prepilin peptidase PilD of P. aeruginosa and BfpP of EPEC (Zhang et al., 1994). Thus, a higher level of specificity may be necessary for complete processing of TcpA compared to that which is necessary for complete processing of bundlin. Regardless of the possible functional differences between TcpJ and BfpP, we contend that the failure of TcpA assembly in EPEC is probably not due to the inability of BfpP to fully process TcpA. The observations reported by Strom et al. (1993) support our contention. They constructed strains of P. aeruginosa that contained mutations in pilD. Although some strains processed as little as 5% of prepilin proteins, they still assembled mature filaments as judged by their susceptibility to killing by the pilus-specific phage PO4. Likewise, we propose that the available amounts of fully processed TcpA in EPEC should be sufficient for subsequent assembly into mature filaments. If so, these observations suggest that constraints which limit TcpA assembly in EPEC occur after production and processing of prepilin subunits. These constraints could result from non-productive interactions occurring between TcpA and existing components encoded by the bfp gene cluster, or from the failure of TcpA to engage with a specific assembly component(s) not present in EPEC. The fact that tcpA in EPEC is permissive to bfpA complementation in trans supports the latter mechanism.

Although similarities exist between the type 4 pilin assembly systems of EPEC and V. cholerae, failure of EPEC to assemble TcpA into mature filaments may be due to several notable differences that exist between these systems. These differences include the possible requirement of a putative minor pilin subunit encoded by tcpB for TCP biogenesis (Manning, 1997) that would be lacking in EPEC. EPEC may lack other functional homologues that are required for TCP assembly in V. cholerae. For example, TcpH, TcpQ and TcpS are all predicted to be periplasmic-associated proteins in V. cholerae, while only BfpU, a highly hydrophilic protein, has been proposed to reside in the periplasm in EPEC (Stone et al., 1996; K. D. Stone & M. S. Donnenberg, unpublished data). Interestingly, the tcp gene cluster also encodes TcpI, a peptide that is highly homologous to the family of methyl-accepting chemotaxis proteins which are involved in environmental sensing (Ogierman et al., 1996); the bfp gene cluster lacks such a homologue. The observation that bundlin is rapidly degraded in the absence of DsbA (Zhang & Donnenberg, 1996), a periplasmic enzyme that mediates disulfide bond formation, contrasts with those of Peek & Taylor (1992), who showed that TCP are still produced in a tcpG (V. cholerae dsbA homologue) mutant. The different effects of these disulfide isomerases on assembly of two similar type 4 pili from these two species could reflect differences in the pilin subunits, differences in the kinetics of pilus biogenesis and disulfide bond formation, or differences in periplasmic proteases that degrade malfolded pilin subunits.

To our knowledge, we have made the first attempt to express and assemble chimeric type 4 pilin subunits from an appropriate host. The first chimera, which possessed the first 58 amino acid residues of bundlin and the last 156 amino acid residues of TcpA, was designed to test whether sequences residing in the N-terminal region of bundlin were sufficient for recognition of chimeric pilin subunits by components of the BFP machinery and, thus, would facilitate their assembly into a functional pilus. The second chimera, a bundlin subunit that contained amino acid residues 120 through 186 of TcpA in place of residues 123 through 183 of bundlin, was designed for two reasons. The first was to determine whether components of the BFP machinery would tolerate changes in a region of the pilin subunit known to contain the most divergent primary structure. The second was to determine whether this region of the chimeric pilin subunit, if assembled into a functional pilus, would confer adhesion properties similar to that of TCP. Both chimeras were carefully designed so that each junction was at equivalent portions of both molecules. Our results indicate that neither chimera was well tolerated in EPEC. Failure to detect either chimera was unlikely to be due to limitations of the antibodies that we used, since regions of either bundlin or TcpA are present in both chimeras and should be recognized by at least one of the polyclonal rabbit antisera. Likewise, failure to detect either chimera was unlikely to be due to inefficient pilin gene expression, since the nucleotide sequences of both gene fusions were identical to that of wild-type bfpA, encoded on pRPA100, except for those regions which encode specific portions of TcpA in place of bundlin. Thus, we conclude that failure to detect either chimeric pilin subunit in EPEC lysates is the result of the degradation of abnormal pilin proteins by specific proteases.

Collectively, our results suggest that the type 4 assembly system of EPEC exhibits some level of specificity with respect to pilin subunit recognition and assembly. Whether this is a unique feature of type 4 pilus biogenesis from EPEC, reflects the limited interchangeability of...
pilin subunits belonging to group B, or is a more general feature of type 4 pilus biogenesis systems other than *Pseudomonas* remains to be determined. As such, it may be difficult to use EPEC as a heterologous host for expression of type 4 pilin subunits derived from different enteric pathogens.

**ACKNOWLEDGEMENTS**

We thank L. Carlson for constructing plasmids pLKC1, pLKCDEL1, -2 and -3, R. Taylor for use of the rabbit polyclonal anti-TcpA peptide 6 antibodies, and R. Anantha for the construction and use of plasmid pRPA100 and the bfpP mutant strain UMD932. We also thank R. Blank and R. Anantha for their helpful comments in reviewing this manuscript. This work was supported by a Public Health Service Award AI37606.

**REFERENCES**


Received 18 May 1999; revised 27 September 1999; accepted 26 November 1999.