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

Abbreviations: BFP, bundle-forming pilus; EAF, EPEC adherence factor; EPEC, enteropathogenic *Escherichia coli*; TCP, toxin-coregulated pilus.

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 composing toxin-coregulated pili (TCP) from *V. cholerae* (Shaw & Taylor, 1990; McKern *et al.*, 1988), 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; Girón *et al.*, 1994), and thin pili encoded by the Inc11 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* (Ogierman *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; Soheli *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 pili, as demonstrated by restoration of both twitching motility and susceptibility to fimbrial-specific bacteriophages. Whether assembly of type 4 pili from a heterologous host is restricted to pili 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 pili 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 *pilT* 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* DH5 α was used to maintain all plasmids except derivatives of the positive-selection suicide vector pCVD442, which were maintained in *E. coli* DH5 α λ pir. Luria-Bertani (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 μ g ampicillin ml $^{-1}$, 50 μ g kanamycin ml $^{-1}$ and 50 μ g 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 μ l volumes containing 100 ng template DNA, 0.1 μ M appropriate primers, 0.2 mM deoxynucleotide triphosphates, 1 $\times$ Thermopol buffer, 4 mM MgSO $_4$ 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 $^{\circ}$ C followed by 29 cycles of 48 $^{\circ}$ C for 45 s, 72 $^{\circ}$ C for 2 min and 95 $^{\circ}$ C for 1 min.

To replace the full-length portion of *bfpA* with *tcpA*, pLKC1 template DNA and primers Donne-36 (5'-CCAAAACGATCTCAAGAAGATCATC-3') and Donne-232 (5'-AGATCTTTTATGAAGTAAACTAGTTAATACGCCCT-3'), which encodes a UAA stop codon (bold faced) and unique BglII and SpeI linker restriction sites (italicized), were used to PCR amplify a 16 kb fragment containing downstream *bfpA*

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
Strains		
<i>E. coli</i>		
E2348/69	Prototype O127:H6 EPEC strain	Levine <i>et al.</i> (1985)
UMD901	E2348/69 <i>bfpAS129C</i>	Zhang & Donnenberg (1996)
UMD932	E2348/69 $\Delta bfpA::tcpA$, <i>bfpP'</i> :: <i>aphA-3</i> ::' <i>bfpP</i>	This study
UMD936	E2348/69 $\Delta bfpA::tcpA$	This study
UMD940	E2348/69 $\Delta bfpA::tcpA$, <i>bfpF'</i> :: <i>aphA-3</i> ::' <i>bfpF</i>	This study
DH5 α	$\Delta lacU169$ <i>recA1 endA1 gyrA96 relA1</i>	Sambrook <i>et al.</i> (1989)
DH5 α <i>ipir</i>	DH5 α (<i>ipir</i>)	Ménard <i>et al.</i> (1993)
<i>V. cholerae</i>		
O395 Sm	Classical O1 Ogawa strain; Sm ^R	Taylor <i>et al.</i> (1987)
BGD4	<i>tcpA</i>	R. Taylor*
Plasmids		
pCR-Script	High-copy-number cloning vector, Cml ^R	Stratagene
pCVD442	Positive-selection suicide vector, Amp ^R	Donnenberg & Kaper (1991)
pJPN101	EAF region II encoding <i>bfpA</i> – <i>L</i> cloned into pJBK68	Nataro <i>et al.</i> (1987)
pLKC1	pACYC184 containing 4.2 kb <i>HindIII</i> fragment from pJPN101	This study
pLKCDEL1	pCR-Script containing 3.4 kb PCR fragment encoding the first 123 residues of BfpA and upstream sequences	This study
pLKCDEL2	pCR-Script containing 1.7 PCR fragment encoding last 11 residues of BfpA and downstream <i>bfp</i> sequences	This study
pLKCDEL3	pLKCDEL2 containing 2.1 kb <i>SpeI</i> – <i>EcoRV</i> fragment from pLKCDEL1; encodes in-frame <i>bfpA</i> deletion	This study
pWKS30	Low-copy-number cloning vector, Amp ^R	Wang & Kushner (1991)
pBPM14	pCR-Script containing 1.6 kb of sequence downstream of <i>bfpA</i>	This study
pBPM15	pCR-Script containing 3.2 kb of sequence upstream of <i>bfpA</i>	This study
pBPM16	pBPM14 containing 3.3 kb <i>NotI</i> – <i>BglII</i> fragment from pBPM15	This study
pBPM17	pBPM16 containing 674 bp <i>NdeI</i> – <i>SpeI</i> fragment from pBPM18	This study
pBPM18	pCR-Script containing 685 bp PCR fragment encoding <i>tcpA</i>	This study
pBPM21	pCVD442 containing <i>tcpA</i> flanked by 1350 bp of upstream and 1050 bp of downstream <i>bfp</i> 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 156 residues of TcpA and downstream <i>bfp</i> sequences	This study
pBPM26	pBPM23 containing 1.6 kb <i>KpnI</i> – <i>SpeI</i> fragment from pBPM22	This study
pBPM27	pWKS30 containing 1 kb <i>BamHI</i> – <i>PstI</i> 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	pLKCDEL3 containing 201 bp <i>SpeI</i> fragment from pBPM33	This study
pBPM35	pWKS30 containing 1 kb <i>BamHI</i> – <i>PstI</i> fragment from pBPM34	This study
pKDS8.2	pCVD442 containing <i>Asp700</i> fragment encoding <i>bfpF</i> :: <i>aphA-3</i>	Anantha <i>et al.</i> (1998)
pRPA100	pWKS30 containing 1 kb <i>BamHI</i> – <i>PstI</i> fragment encoding <i>bfpA</i> and <i>bfpG'</i>	R. Anantha, University of Maryland

* Dept of Microbiology, Dartmouth Medical School, USA.

sequences. Similarly, primers Donne-26 (5'-AACGCGGGG-AGGCAGACA-3') and Donne-233 (5'-AGATCTAACCAT-ATGAAGTGTTCCTTAT-3'), which encode an AUG start codon (bold faced) and unique *NdeI* and *BglII* linker restriction sites (italicized), were used to PCR amplify a 3.2 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

with primers Donne-250 (5'-GCGGACTAGTTTAGCTGT-TACCAAATGCACCGCC-3'), which encodes a UAA stop codon (bold faced) and a unique *SpeI* restriction site (italicized), and Donne-251 (5'-GGCGCATATGCAATTAT-TAAAACAGC-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 encoding *tcpA* 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 *SacI*, 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'-ACTAGTTGCGCT-CATTACTTCTG-3') and Donne-294 (5'-TGAGTGTCT-GCTCACTACAG-3') were used to amplify a 1.5 kb fragment from pLKCl. 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'-ACT-AGTCAAGTTGCACTGACACAGAC-3') and Don-1 (5'-AGAATCACTGGAAGTAC-3') to amplify a 1.6 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 pWKS30 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 pLKDEL3, a plasmid that encodes an in-frame deletion of codons 123 through 183 in bundlin. Briefly, pLKCl and primers Donne-21 and Donne-26 (5'-GCCACTAGTAA-GCGTCAGCTAGTAACCAA-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'-GCCACTAGTACCAATAAA-GTTACATAT-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 pLKDEL1 and pLKDEL2, respectively. A 2.1 kb *SpeI-EcoRV* fragment was excised from pLKDEL1 and cloned into corresponding sites of pLKDEL2 to create pLKDEL3. Primers Donne-327 (5'-GGACTAGTTGCAAGACACTTA-TTACCAG-3') and Donne-311 (5'-ACTAGTACATAATTT-CTCAACGTGAGTG-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 pLKDEL3 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 DH5 α *pir* 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 DH5 α *pir* 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 *Taq* DNA polymerase in 100 μ l volumes. Reactions were subjected to initial denaturation of 2 min at 94 °C followed by 29 cycles of 54 °C for 45 s, 72 °C for 2 min and 95 °C for 1 min. Primers Donne-5 (5'-AAAC-AGGATACCAAAAGA-3') and Donne-6 (5'-TCTTGGTG-CTTGCGTGTGTC-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'-GGGAATTCCTGATTCCGTGTGATATCATG-3') and Donne-236 (5'-GGGGATCCTGCATAATATTTTAGCT-AATCAGGTT-3') were used to amplify *bfpF*. Plasmid pRPA100 was electroporated into competent 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 μ l 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 OD₆₀₀) 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 6.8; 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 semidry Multiphor II NovaBlot transfer apparatus (Pharmacia) as described by the manufacturer. Membranes were blocked initially with PBS containing 0.1% (v/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 μ l) 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-

logous type 4 pili encoded by *tcpA*, we amplified the entire *tcpA* gene from *V. cholerae* O395 and cloned it into the *bfp* gene cluster in place of the entire *bfpA* gene (Fig. 1). Plasmid pBPM17, which encodes *tcpA* and flanking *bfp* gene cluster sequences, was mobilized into wild-type EPEC by triparental conjugation. The *sacB* gene on pBPM21 was used to select for allelic exchange of full-length *tcpA* for full-length *bfpA*, creating strain UMD936. Similarly, plasmid pKDS8.2 was mobilized into strain UMD936 as a means of introducing an *aphA*-3-marked *bfpF* mutation to create strain UMD940. Since cells of EPEC containing a mutation in *bfpF* are hyperfimbriate, we hypothesized that introduction of this mutation would facilitate our ability to detect type 4 pili encoded by *tcpA* from EPEC. PCR analysis of wild-type EPEC, UMD936 and UMD940 confirmed the identities of *bfpA*, *tcpA* and *bfpF*::*aphA*-3, respectively, based upon the expected size differences of resultant PCR products (data not shown). In addition, sequence analysis of PCR products amplified from cells of UMD936 indicated that no mutations were present in *tcpA* and flanking sequence.

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 auto-aggregation 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 *bfpA*, 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. 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 *Bam*HI-*Pst*I fragment containing *bfpA* and *bfpG'* cloned into the low-copy-number plasmid pWKS30. One-kilobase *Bam*HI-*Pst*I fragments containing gene fusions, which encode chimeric pilin subunits possessing either 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 pWKS30 to create plasmids pBPM27 and pBPM35, respectively. Filled areas indicate nucleotide sequences that encode corresponding regions of *TcpA*.

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 ($\Delta tcpA$), wild-type EPEC E2348/69, UMD901 (*bfpAS129C*), UMD932 ($\Delta bfpP::aphA-3::bfpP$), UMD936 ($\Delta bfpA::tcpA$) and UMD940 ($\Delta bfpA::tcpA$, *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.

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. 4. Electron microscopy examination of type 4 pilus structures expressed by wild-type and recombinant EPEC strains. Strains were grown under conditions for optimal expression of BFP, spotted onto Formvar-carbon-coated copper grids, stained with phosphotungstic acid and analysed by transmission electron 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. 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 *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 bundlin 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 *bfpA* and upstream promoter sequences, except for those regions that encode specific portions of TcpA in place of bundlin. 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-bundlin (Fig. 3d) antibodies were used. As expected, processed bundlin was detected by anti-bundlin 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 *P. aeruginosa* can assemble fimbrial subunits of *D. nodosus*, *M. bovis* and *N. gonorrhoeae* into mature filaments on the cell surface (Watson *et al.*, 1996; Hoyne *et al.*, 1992; Beard *et al.*, 1990; Mattick *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 *V. cholerae*, were unsuccessful. Cells of UMD936, the EPEC recombinant strain containing the full replacement of *bfpA* with *tcpA*, consistently failed to assemble type 4 pili as judged by their failure to autoaggregate in culture, adhere to HEp-2 cells *in vitro*, and assemble any type 4 pilin structures. Two observations suggest that this failure was not due to the manner in which the *bfpA* gene was replaced with *tcpA in situ*. First, this replacement was precise, as no other sequences within the *bfp* gene cluster were altered other than those which encoded bundlin. Second, reintroduction of *bfpA*, encoded on pRPA100, into cells of UMD936 resulted in their ability to perform localized adherence, autoaggregate and assemble processed bundlin into mature filaments. Thus, we conclude that the assembly machinery encoded by the *bfp* gene cluster in UMD936 is functionally intact, but is incapable of assembling type 4 pili composed of TcpA. We also examined whether a *bfpF* mutation would facilitate expression of type 4 pili encoded by *tcpA* from EPEC since cells of a *bfpF* mutant, like those of a *pilT* mutant, produce many more bacterial filaments than do wild-type cells (Bieber *et al.*, 1998; Bradley, 1974). Similar to our earlier results, cells of strain UMD940 containing *bfpF* and the *tcpA* substitution also failed to autoaggregate in culture, adhere to HEp-2 cells *in vitro*, and assemble any detectable surface structures resembling type 4 pili. Thus, failure to detect any type 4 pilin structures encoded by *tcpA* from cells of UMD936 would not necessarily be the result of our limits of detection.

EPEC and *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 *tcpA* from *V. cholerae*. Genes required for TCP or BFP biogenesis appear to be organized or clustered in a single operon (Ramer *et al.*, 1996; Brown & Taylor, 1995). Both gene clusters begin with the gene encoding the major structural subunit, *tcpA* and *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 (Ogierman *et al.*, 1993; Soheli *et al.*, 1996; Stone *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 VcpD, 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 pili 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 malformed 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 pili 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.

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REFERENCES

- Anantha, R. P., Stone, K. D. & Donnenberg, M. S. (1998). The role of BfpF, a member of the PilT family of putative nucleotide-binding proteins, in type IV pilus biogenesis and in interactions between enteropathogenic *Escherichia coli* and host cells. *Infect Immun* **66**, 122–131.
- Beard, M. K., Mattick, J. S., Moore, L. J., Mott, M. R., Marrs, C. F. & Egerton, J. R. (1990). Morphogenetic expression of *Moraxella bovis* fimbriae (pili) in *Pseudomonas aeruginosa*. *J Bacteriol* **172**, 2601–2607.
- Bieber, D., Ramer, S. W., Wu, C. Y., Murray, W. J., Tobe, T., Fernandez, R. & Schoolnik, G. K. (1998). Type IV pili, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. *Science* **280**, 2114–2118.
- Bradley, D. E. (1974). The adsorption of *Pseudomonas aeruginosa* pilus-dependent bacteriophages to a host mutant with non-retractile pili. *Virology* **58**, 149–163.
- Brown, R. C. & Taylor, R. K. (1995). Organization of *tcp*, *acf*, and *toxT* genes within a ToxT-dependent operon. *Mol Microbiol* **16**, 425–439.
- Donnenberg, M. S. & Kaper, J. B. (1991). Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect Immun* **59**, 4310–4317.
- Donnenberg, M. S. & Nataro, J. P. (1995). Methods for studying adhesion of diarrheagenic *Escherichia coli*. *Methods Enzymol* **253**, 324–336.
- Donnenberg, M. S., Girón, J. A., Nataro, J. P. & Kaper, J. B. (1992). A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. *Mol Microbiol* **6**, 3427–3437.
- Donnenberg, M. S., Yu, J. & Kaper, J. B. (1993). A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. *J Bacteriol* **175**, 4670–4680.
- Girón, J. A., Levine, M. M. & Kaper, J. B. (1994). Longus: a long pilus ultrastructure produced by human enterotoxigenic *Escherichia coli*. *Mol Microbiol* **12**, 71–82.
- Hahn, H. P. (1997). The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa* – a review. *Gene* **192**, 99–108.
- Harlow, D. H. & Lane, D. (1988). *Antibodies: a Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Herrington, D. A., Hall, R. H., Losonsky, G., Mekalanos, J. J., Taylor, R. K. & Levine, M. M. (1988). Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. *J Exp Med* **168**, 1487–1492.
- Hoynes, P. A., Haas, R., Meyer, T. F., Davies, J. K. & Elleman, T. C. (1992). Production of *Neisseria gonorrhoeae* pili (fimbriae) in *Pseudomonas aeruginosa*. *J Bacteriol* **174**, 7321–7327.
- Johnson, K., Parker, M. L. & Lory, S. (1986). Nucleotide sequence and transcriptional initiation site of two *Pseudomonas aeruginosa* pilin genes. *J Biol Chem* **261**, 15703–15708.
- Kaufman, M. R., Seyer, J. M. & Taylor, R. K. (1991). Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram-negative bacteria. *Genes Dev* **5**, 1834–1846.
- Kim, S. R. & Komano, T. (1997). The plasmid R64 thin pilus identified as a type IV pilus. *J Bacteriol* **179**, 3594–3603.
- Koomey, M. (1995). Prepilin-like molecules in type 4 pilus biogenesis: minor subunits, chaperones or mediators of organelle translocation? *Trends Microbiol* **3**, 409–411.
- Lepper, A. W. D., Atwell, J. L., Lehrbach, P. R., Schwartzkoff, C. L., Egerton, J. R. & Tennent, J. M. (1995). The protective efficacy of cloned *Moraxella bovis* pili in monovalent and multivalent vaccine formulations against experimentally induced infectious bovine keratoconjunctivitis (IBK). *Vet Microbiol* **45**, 129–138.
- Levine, M. M., Nataro, J. P., Karch, H. M., Baldini, M., Kaper, J. B., Black, R. E., Clements, M. L. & O'Brien, A. D. (1985). The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. *J Infect Dis* **152**, 550–559.
- McKern, N. M., Stewart, D. J. & Strike, P. M. (1988). Amino acid sequences of pilins from serologically distinct strains of *Bacteroides nodosus*. *J Protein Chem* **7**, 157–164.
- Manning, P. A. (1997). The *tcp* gene cluster of *Vibrio cholerae*. *Gene* **192**, 63–70.
- Marrs, C. F., Schoolnik, G., Koomey, J. M., Hardy, J., Rothbard, J. & Falkow, S. (1985). Cloning and sequencing of a *Moraxella bovis* pilin gene. *J Bacteriol* **163**, 132–139.
- Marsh, J. W. & Taylor, R. K. (1998). Identification of the *Vibrio cholerae* type 4 prepilin peptidase required for cholera toxin secretion and pilus formation. *Mol Microbiol* **29**, 1481–1492.
- Mattick, J. S., Bills, M. M., Anderson, B. J., Dalrymple, B., Mott, M. R. & Egerton, J. R. (1987). Morphogenetic expression of *Bacteroides nodosus* fimbriae in *Pseudomonas aeruginosa*. *J Bacteriol* **169**, 33–41.
- Ménard, R., Sansonetti, P. J. & Parsot, C. (1993). Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J Bacteriol* **175**, 5899–5906.
- Meyer, T. F., Billyard, E., Haas, R., Storzach, S. & So, M. (1984). Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. *Proc Natl Acad Sci USA* **81**, 6110–6114.
- Nataro, J. P., Maher, K. O., Mackie, P. & Kaper, J. B. (1987). Characterization of plasmids encoding the adherence factor of enteropathogenic *Escherichia coli*. *Infect Immun* **55**, 2370–2377.
- Ogierman, M. A., Zabihi, S., Mourtziros, L. & Manning, P. A. (1993). Genetic organization and sequence of the promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. *Gene* **126**, 51–60.
- Ogierman, M. A., Voss, E., Meaney, C., Faast, R., Attridge, S. R. & Manning, P. A. (1996). Comparison of the promoter proximal regions of the toxin-co-regulated *tcp* gene cluster in classical and El Tor strains of *Vibrio cholerae* O1. *Gene* **170**, 9–16.

- Parge, H. E., Forest, K. T., Hickey, M. J., Christensen, D. A., Getzoff, E. D. & Tainer, J. A. (1995). Structure of the fibre-forming protein pilin at 2.6 Å resolution. *Nature* **378**, 32–38.
- Peek, J. A. & Taylor, R. K. (1992). Characterization of a periplasmic thiol:disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. *Proc Natl Acad Sci USA* **89**, 6210–6214.
- Potts, W. J. & Saunders, J. R. (1988). Nucleotide sequence of the structural gene for class I pilin from *Neisseria meningitidis*: homologies with the *pilE* locus of *Neisseria gonorrhoeae*. *Mol Microbiol* **2**, 647–653.
- Ramer, S. W., Bieber, D. & Schoolnik, G. K. (1996). BfpB, an outer membrane lipoprotein required for the biogenesis of bundle-forming pili in enteropathogenic *Escherichia coli*. *J Bacteriol* **178**, 6555–6563.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shaw, C. E. & Taylor, R. K. (1990). *Vibrio cholerae* O395 *tcpA* pilin gene sequence and comparison of predicted protein structural features to those of type 4 pilins. *Infect Immun* **58**, 3042–3049.
- Sohel, I., Puente, J. L., Murray, W. J., Vuopio-Varkila, J. & Schoolnik, G. K. (1993). Cloning and characterization of the bundle-forming pilin gene of enteropathogenic *Escherichia coli* and its distribution in *Salmonella* serotypes. *Mol Microbiol* **7**, 563–575.
- Sohel, I., Puente, J. L., Ramer, S. W., Bieber, D., Wu, C.-Y. & Schoolnik, G. K. (1996). Enteropathogenic *Escherichia coli*: identification of a gene cluster coding for bundle-forming pilus morphogenesis. *J Bacteriol* **178**, 2613–2628.
- Stone, K. D., Zhang, H.-Z., Carlson, L. K. & Donnenberg, M. S. (1996). A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for biogenesis of a type IV pilus. *Mol Microbiol* **20**, 325–337.
- Strom, M. S. & Lory, S. (1993). Structure–function and biogenesis of the type IV pili. *Annu Rev Microbiol* **47**, 565–596.
- Strom, M. S., Nunn, D. N. & Lory, S. (1993). A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. *Proc Natl Acad Sci USA* **90**, 2404–2408.
- Sun, D., Lafferty, M. J., Peek, J. A. & Taylor, R. K. (1997). Domains within the *Vibrio cholerae* toxin coregulated pilin subunit that mediate bacterial colonization. *Gene* **192**, 79–85.
- Taniguchi, T., Fujino, Y., Yamamoto, K., Miwatani, T. & Honda, T. (1995). Sequencing of the gene encoding the major pilin of pilus colonization factor antigen III (CFA/III) of human enterotoxigenic *Escherichia coli* and evidence that CFA/III is related to type IV pili. *Infect Immun* **63**, 724–728.
- Taylor, R. K., Miller, V. L., Furlong, D. B. & Mekalanos, J. J. (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci USA* **84**, 2833–2837.
- Voss, E., Manning, P. A. & Attridge, S. R. (1996). The toxin-coregulated pilus is a colonization factor and protective antigen of *Vibrio cholerae* El Tor. *Microb Pathog* **20**, 141–153.
- Vuopio-Varkila, J. & Schoolnik, G. K. (1991). Localized adherence by enteropathogenic *Escherichia coli* is an inducible phenotype associated with the expression of new outer membrane proteins. *J Exp Med* **174**, 1167–1177.
- Wang, R. F. & Kushner, S. R. (1991). Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**, 195–199.
- Watson, A. A., Mattick, J. S. & Alm, R. A. (1996). Functional expression of heterologous type 4 fimbriae in *Pseudomonas aeruginosa*. *Gene* **175**, 143–150.
- Whitchurch, C. B., Hobbs, M., Livingston, S. P., Krishnapillai, V. & Mattick, J. S. (1991). Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene* **101**, 33–44.
- Zhang, H.-Z. & Donnenberg, M. S. (1996). DsbA is required for stability of the type IV pilin of enteropathogenic *Escherichia coli*. *Mol Microbiol* **21**, 787–797.
- Zhang, H.-Z., Lory, S. & Donnenberg, M. S. (1994). A plasmid-encoded prepilin peptidase gene from enteropathogenic *Escherichia coli*. *J Bacteriol* **176**, 6885–6891.

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