BfpL is essential for type IV bundle-forming pilus biogenesis and interacts with the periplasmic face of BfpC

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Enteropathogenic *Escherichia coli* (EPEC) causes diarrhoea among infants in developing countries. The bundle-forming pilus (BFP), a type IV pilus found on the surface of EPEC, is essential for full virulence of typical EPEC strains. The machinery for BFP assembly and function is encoded by an operon of 14 genes. Here we investigate the role in pilus biogenesis of BfpL, a small protein with a single N-terminal predicted transmembrane domain reminiscent of pilin-like proteins. We confirmed that a *bfpL* mutant lacks BFP, and associated auto-aggregation and localized adherence phenotypes. Furthermore, we found that a double mutant unable to express both the putative retraction ATPase BfpF and BfpL also lacks BFP and associated phenotypes, distinguishing BfpL from pilin-like proteins. Western blots of sheared pilus preparations did not suggest that BfpL is a component of BFP. Topology studies using C-terminal truncations and a dual reporter revealed that most of the BfpL protein resides in the periplasm. Further, we demonstrated through yeast two-hybrid assays and confirmed by fluorescence anisotropy that BfpL interacts with the periplasmic face of BfpC. Thus, BfpL has a function distinct from those of pilin-like proteins and is instead part of an inner-membrane subassembly complex that is believed to extract bundlin, the main pilus subunit, from the inner membrane to be incorporated into BFP.

INTRODUCTION

Type IV pili (T4P), the most widespread class of pili known, are produced by numerous Gram-negative and Gram-positive bacteria (Proft & Baker, 2009) and archaea (Fröls *et al.*, 2008; Ng *et al.*, 2011). They are closely related to type II secretion (T2S) systems (Pelicic, 2008). T4Ps are highly flexible and extremely thin, ranging from 5 to 8 nm in diameter, yet capable of growing to several micrometres in length. For many bacterial pathogens, T4P play important roles in disease, functioning as adhesins and colonization factors (Bieber *et al.*, 1998; Saiman & Prince, 1993; Stone & Abu Kwaik, 1998; Tacket *et al.*, 1998; Taha *et al.*, 1998). T4P systems are subdivided into two classes by amino acid sequence similarity and the length of the main pilus subunit or pilin (Craig *et al.*, 2004). In comparison

with type IVa pilins, type IVb pilins have longer leader sequences, are larger and more complex, and have been found predominantly on bacteria colonizing the intestine. Current models indicate that pilin subunits are polymerized into a left-handed three-start helical structure with the N-terminal spine buried in the filament core. Portions of the globular head domain are exposed on the pilus surface. However, the actual assembly process remains obscure. T4P from some species intertwine with one another, while those from other species remain as individual fibres.

Enteropathogenic *Escherichia coli* (EPEC) is a common cause of diarrhoea in children under the age of 2 years in the developing world (Donnenberg, 2002). Typical strains of EPEC adhere to host cells in clusters, a phenomenon called localized adherence (LA), which requires a T4bP called the bundle-forming pilus (BFP) (Donnenberg *et al.*, 1992). BFP are encoded by the 14-gene *bfp* operon (Sohel *et al.*, 1996; Stone *et al.*, 1996). The proteins encoded by this operon include several that are common to all Gram-negative T4P systems: the pilin, known as bundlin for BFP (Ramboarina *et al.*, 2005); BfpP, a prepilin peptidase that removes the T4P signal sequence and N-methylates the

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Abbreviations: BFP, bundle-forming pilus/i; EPEC, enteropathogenic *Escherichia coli*; FPLC, fast protein liquid chromatography; IM, inner membrane; LA, localized adherence; mP, millipolarization units; T2S, type II secretion; T4P, type IV pilus/i; TEM, transmission electron microscopy.

nascent N terminus of bundlin (Donnenberg, 2012; Zhang *et al.*, 1994); BfpI, BfpJ and BfpK, referred to as pilin-like proteins, which contain T4P signal sequences and are also processed to their mature forms by BfpP (Ramer *et al.*, 2002); BfpB, the secretin, a lipoprotein that is presumed to form the outer-membrane pore through which the pili extend (Ramer *et al.*, 1996); BfpD, a hexameric ATPase required for pilus extension (Yamagata *et al.*, 2012); BfpF, a putative ATPase required for phenotypes attributed to pilus retraction (Anantha *et al.*, 1998; Bieber *et al.*, 1998); and BfpE, a polytopic inner-membrane (IM) protein (Blank & Donnenberg, 2001).

In addition to the above proteins, which all have recognizable sequence similarities to proteins from other T4P biogenesis systems, several other proteins are required for BFP biogenesis, but appear to be specific. BfpG and BfpU are found primarily in the periplasm, but both are recruited to the outer membrane by BfpB (Daniel *et al.*, 2006) and BfpU can also be detected in the cytoplasm (Schreiber *et al.*, 2002). BfpC is a bitopic IM protein that interacts closely with BfpD, BfpE and BfpF (Milgotina *et al.*, 2011). Recently, the cytoplasmic N-terminal BfpC (N-BfpC) crystal structure was solved, and shares a common fold with the cytoplasmic T4aP component PilM (Karuppiah & Derrick, 2011) and, surprisingly, resembles more strongly the N terminus of T2S component EpsL (N-EpsL) (Abendroth *et al.*, 2004a), despite a complete lack of sequence conservation between N-BfpC and either protein (Yamagata *et al.*, 2012). Thus, the lack of sequence similarities belies the structural conservation among these proteins. As BfpC and BfpE recruit the ATPase BfpD to the membrane, BfpC is also a functional homologue of EpsL (Abendroth *et al.*, 2005; Sandkvist *et al.*, 1995). In addition to interactions of N-EpsL with the ATPase, cross-linking experiments indicate that EpsL interacts with EpsG, the main T2S pilin-like protein, possibly acting as a scaffold to link the two (Gray *et al.*, 2011).

At the end of the *bfp* operon is a protein of unknown function, BfpL, which does not appear to have sequence homologues in any known T4P system. Prior studies have indicated that a *bfpL* mutant fails to make BFP and that BfpL

fractionates predominantly with the IM (Ramer *et al.*, 2002). Furthermore, in the absence of BfpL, less BfpJ could be detected, while mutations of *bfpI*, *bfpJ* or *bfpK* were associated with a decreased abundance of BfpL. These results were considered to be strongly suggestive of an interaction between BfpL and these pilin-like proteins. Intriguingly, while BfpL is not processed by BfpP (Ramer *et al.*, 2002), BfpL bears some similarity to pilin-like proteins, including a single stretch of hydrophobic amino acids near the N terminus predicted to represent a transmembrane domain and several amino acids nearly identical to a prepilin peptidase cleavage site (Fig. 1).

Experiments in *Neisseria gonorrhoeae*, *Neisseria meningitidis* and *Pseudomonas aeruginosa* (Carbonnelle *et al.*, 2006; Giltner *et al.*, 2010; Winther-Larsen *et al.*, 2005) have demonstrated that pilin-like proteins are only essential for T4P biogenesis when the putative retraction ATPase protein is also present. Thus, T4P are made by bacteria that have null mutations in the genes for pilin-like proteins if the gene for the retraction ATPase is also mutated. Synthesis of T4P alone in the absence of pilus retraction has been interpreted as evidence that pilin-like proteins play an ancillary role in biogenesis, either facilitating extension or hindering retraction. To test the hypothesis that BfpL is functionally and structurally similar to pilin-like proteins, we constructed in-frame deletions of the *bfpL* gene in both a wild-type EPEC background and a *bfpF* mutant background. We examined these mutants for BFP expression by immunofluorescence microscopy and transmission electron microscopy (TEM), and for associated auto-aggregation and LA phenotypes. We examined the localization and topology of BfpL, conducted a series of yeast two-hybrid assays to screen for interactions between BfpL and other proteins in the BFP assembly apparatus, and used fluorescence anisotropy to test the hypothesis that BfpL binds to the periplasmic C terminus of BfpC.

METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. Mutations in *bfpL* were

BfpA	VSKIMNKYE	KGLSLIESAM	VLALAATVTA	GVMFYQSAS	DSNKSQNAIS	51
pred	Iiiiiiii	ihhhhhhhh	hhhhhhhhh	HHHhoooo	oooooooO	
BfpI	MFILRNKRKE	SGLSLIEASL	VIILSGLVIV	LVQSAISKII	KYAVHENALH	50
pred	Iiiiiiii	ihhhhhhhh	hhhhhhhhh	Hooooooo	oooooooO	
BfpJ	MIRKE	SGLSLIEVMI	GVLFFSIVSL	SVYNYNRS	EQHYMNVSV	45
pred	Iiiii	HHHHHHHH	HHHHHHHH	ooooooo	ooooO	
BfpK	MIFEGRQ	KGLSLIEIAM	VIILSVVVIS	GGSYIISLFK	VFAQSCVAT	48
pred	Iiiiiiii	ihhhhhhhh	hhhhhhhhh	HHHHHHHh	ooooooo	
BfpL	MLFFWC	GFFSLIVSIL	NISMGYNDRL	FLDEQNRIK	ENIYVDYLT	46
pred	IiiiiH	HHHHHHHH	HHHHHH	ooooooo	ooooO	

Fig. 1. Alignment of the N-terminal regions of bundlin (BfpA); the pilin-like proteins BfpI, BfpJ and BfpK; and BfpL. BfpL shares three of the six amino acids (underlined and in grey type) near the prepilin peptidase cleavage site found in bundlin, BfpI, BfpJ and BfpK (in grey type). Protein names are in bold type. The predicted membrane topology (pred) of each protein as determined by HMMTOP version 2.0 is also shown (Tusnady & Simon, 2001). An upper-case I indicates an amino acid predicted to reside in the cytoplasm, a lower-case i indicates a predicted inner tail, an upper-case H denotes a transmembrane helix, a lower-case o denotes an outer tail, i.e. in the periplasmic region, and an upper-case O denotes a periplasmic domain.

Table 1. Strains, and plasmids used in this study

Strain or plasmid	Description or genotype	Reference or source
Strains		
E2348/69	Wild-type EPEC strain, serotype O127:H6	Levine <i>et al.</i> (1978)
UMD916	E2348/69 <i>bfpF::aphA3</i>	Anantha <i>et al.</i> (1998)
UMD955	E2348/69 $\Delta bfpL$	This study
UMD961	UMD916 $\Delta bfpL$	This study
BL21(DE3) pLysS	F ⁻ , <i>dcm</i> , <i>ompT</i> , <i>hsdS</i> (r _B ⁻ m _B ⁻)	Novagen
AH 109	<i>Saccharomyces cerevisiae</i> auxotrophic strain used in yeast two-hybrid assays	Clontech
Plasmids		
pPF302	Expression vector for His–bundlin with DsbA signal sequence	Fernandes <i>et al.</i> (2007)
pEM87	Expression vector for His–BfpC–C terminus with DsbA signal sequence	This study
pLDPF-L	Expression vector for His–BfpL with DsbA signal sequence	This study
pBAD33	Cloning vector with multiple cloning site (MCS) under control of an <i>araC</i> arabinose-inducible promoter	Guzman <i>et al.</i> (1995)
pLDBAD-L	pBAD33 complementation vector with <i>bfpL</i> cloned into MCS	This study
pTE120	Dual reporter vector used for topology studies	Blank & Donnenberg (2001)
pWS42	Dual reporter vector with codons for BfpL amino acids 1–15 inserted into MCS	This study
pWS49	Dual reporter vector with codons for BfpL amino acids 1–35 inserted into MCS	This study
pWS44	Dual reporter vector with codons for BfpL amino acids 1–186 inserted into MCS	This study
pWS45	Dual reporter vector with codons for BfpL amino acids 1–159 inserted into MCS	This study
pRPA311	Dual reporter vector with codons for BfpC amino acids 1–164 inserted into MCS	Crowther <i>et al.</i> (2004)
pRPA312	Dual reporter vector with codons for BfpC amino acids 1–188 inserted into MCS	Crowther <i>et al.</i> (2004)
pGADT7	Yeast two-hybrid vector, contains GAL4 activation domain, functions as a negative control when combined with any partner	Clontech
pGBKT7	Yeast two-hybrid vector, contains GAL4 DNA-binding domain, functions as a negative control when combined with any partner	Clontech
pGADT7-T	Yeast two-hybrid vector with T-antigen cloned into pGADT7, when combined with pGBKT7-p53 it functions as the only positive control provided in the yeast two-hybrid system, functions as a negative control when combined with any other partner	Clontech
pGBKT7-Lam	Yeast two-hybrid vector with laminin cloned into pGBKT7, functions as a negative control, since it has no putative binding partner	Clontech
pGBKT7-p53	Yeast two-hybrid vector with p53 cloned into pGBKT7, when combined with pGADT7-T it functions as the only positive control provided in the yeast two-hybrid system	Clontech
pASADL1	Yeast two-hybrid vector with soluble BfpL (amino acids 31–149) cloned into pGADT7	This study
pASBDL2	Yeast two-hybrid vector with soluble BfpL (amino acids 31–149) cloned into pGBKT7	This study
pEMADNC	Yeast two-hybrid vector with cytoplasmic N terminus of BfpC (amino acids 1–164) cloned into pGADT7	This study
pEMBDNC	Yeast two-hybrid vector with cytoplasmic N terminus of BfpC (amino acids 1–164) cloned into pGBKT7	This study
pEMADC7	Yeast two-hybrid vector with periplasmic C terminus of BfpC (amino acids 188–402) cloned into pGADT7	This study
pEMBDC8	Yeast two-hybrid vector with periplasmic C terminus of BfpC (amino acids 188–402) cloned into pGBKT7	This study
pEMADA3	Yeast two-hybrid vector with soluble bundlin (amino acids 25–180) cloned into pGADT7	This study
pEMBDA4	Yeast two-hybrid vector with soluble bundlin (amino acids 25–180) cloned into pGBKT7	This study

engineered to delete all but the first five and last five codons separated by an 85 nt ‘scar’ sequence as described by Datsenko & Wanner (2000). Oligonucleotide primers are listed in Table 2. Complementation plasmids were created by amplifying each gene including its predicted ribosome-binding site with PFX polymerase and cloning into the *SacI* and *XbaI* restriction sites of the pBAD33 vector. The correct DNA sequences were confirmed in all plasmids.

Expression and purification of BfpL. For BfpL purification, the first 19 amino acids, which are predicted to be highly hydrophobic,

were excluded, codons optimized for *E. coli* were generated by reverse translation and the resulting gene was synthesized commercially (GeneDesign, Inc., Osaka, Japan; http://www.saito.tv/e/lsp/LSP_GuideList/English/GeneDesign.htm?m3). Expression plasmid pLDPF-L was created by cloning optimized *bfpL* into pPF302 (Fernandes *et al.*, 2007), a modified version of pET39b+ containing the DsbA signal sequence immediately upstream of an N-terminal hexahistidine tag. An overnight culture of *E. coli* strain BL21(DE3) containing pLDPF-L was diluted 1:50 in 1 l Luria broth (LB) containing 50 µg kanamycin ml⁻¹ and grown in a 30 °C shaker at 225 r.p.m. until it reached OD₆₀₀ 0.5.

Table 2. Primers used in this study

Primer	Sequence (5'→3')*
λ-Red Recomb. BfpL FWD	cacagggcttctgttaaacggtactagtattgttattcttgggtgtaggctggagctgcttc
λ-Red Recomb. BfpL REV	tagcctaaaaaactacagttctggtggtctatcccggtttgttgacatgaatatcctccttag
BfpL Comp. for pLDBAD-L FWD	gaattcaaacggtactagtattgttattcttctgg
BfpL Comp. for pLDBAD-L REV	ccgggctatccgtttgttgaa
BfpC Dual Reporter FWD	ggccatggtaagaataatcttgg
BfpC1-164 Dual Reporter REV	ccggatccctcgagttttccttaatttctccc
BfpC1-188 Dual Reporter REV	ggctcgagattatataatgatgacaaag
BfpL Dual Reporter FWD	ccacatgttattcttctggtgtggtttt
BfpL1-15 Dual Reporter REV	tcgagaatggatacaattaatgaaaaaaaccacaccagaagaataa
BfpL1-35 Dual Reporter REV	cgggatccctcgagaataatcggtttgttcatcgag
BfpL1-86 Dual Reporter REV	cgggatccctcgagattatcatatccagacaatttatctt

*Underlined type represents sequences from the cassette used for mutagenesis.

Protein expression was then induced with IPTG. The culture was incubated for an additional 1 h in the 30 °C shaker and pelleted by centrifugation for 10 min at 5000 *g*. Proteins from inclusion bodies were isolated as described elsewhere (Caltech.edu; <http://www.its.caltech.edu/~bjorker/Protocols/Isolation%20of%20proteins%20from%20.pdf>), with the following modifications: no DTT was added to any solution and the final pellet containing inclusion bodies was resuspended in 8 M urea, 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0. Resuspended protein solutions were applied to a Qiagen nickel-nitrilotriacetic acid (NTA) agarose resin column and washed repeatedly with resuspension buffer. Protein was eluted by decreasing the pH of the resuspension solution by pH 0.5 increments to a final pH of 5.0. Samples containing purified denatured histidine-tagged BfpL (His-BfpL) were refolded by adding 1 ml of the protein solution drop-wise to 200 ml buffer containing 100 mM Tris/HCl, pH 8.0, 400 mM L-arginine, 2 mM disodium EDTA, 0.5 mM oxidized glutathione and 5 mM reduced glutathione. Samples were slowly stirred at 4 °C for 8 h or overnight. The process was repeated by adding successive 1 ml samples of purified denatured BfpL solution until all protein was dissolved in refolding buffer. Refolded samples were concentrated to 5 ml using Millipore Amicon Ultra-15 filtration units.

Size exclusion chromatography. To examine the secondary structure of BfpL, refolded and unfolded samples of BfpL were analysed using a Jasco J-800 CD spectrometer. In addition, protein samples were loaded onto a Sephacryl S-100 column (GE Healthcare Life Sciences) equilibrated with 50 mM NaH₂PO₄, 300 mM NaCl, pH 7.4 (size exclusion buffer), using an AKTA Prime fast protein liquid chromatography (FPLC) system. Size exclusion buffer was run over the column at a rate of 0.5 ml min⁻¹ and fractions were collected at 10 min intervals, with data on the peak UV expression over time analysed by the AKTA Primeview Evaluation 1.0 program. Fractions corresponding to observed peaks were analysed by Western blotting using affinity-purified rat polyclonal anti-BfpL serum.

Expression and purification of the C terminus of BfpC. A 100 ml overnight culture of BL21(DE3) cells containing pEM87, a plasmid in which codons 188–402 of *bfpC* replaced those of *bfpA* in pPF302 as described above, was diluted 1:100 in 1.5 l LB with 50 µg kanamycin ml⁻¹. The culture was incubated at 25 °C and 150 r.p.m. until it reached OD₆₀₀ 0.3, then induced with 0.4 mM IPTG and incubated for an additional 3 h at 25 °C. Cells were pelleted by centrifugation at 5000 *g* for 10 min, then resuspended in wash buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0. Cells were lysed using a French press at 20 000 p.s.i. (138 MPa), then centrifuged at 35 000 *g* for 35 min. The supernatant was applied to a nickel-NTA agarose resin column and washed three times with wash buffer, then once

with wash buffer containing 5 mM imidazole. Proteins were eluted with wash buffer containing 250 mM imidazole. Fractions containing His-BfpC-C were dialysed twice in wash buffer, then loaded onto a HiTrap high-performance chelating column charged with NiSO₄. The column was attached to an AKTA Prime FPLC system and a gradient consisting of wash buffer with gradually increasing concentrations of imidazole up to 500 mM was applied. Fractions containing His-BfpC-C were combined into a 3 ml sample with a concentration of 30–50 µM.

Cellular fractionation and pilus shear preparations. To confirm the cellular location of BfpL we first isolated membrane and soluble fractions in the following manner: 2.5 ml of an overnight LB culture of E2348/69 was inoculated in 500 ml Dulbecco's Modified Eagle's Medium (DMEM) and grown in a 37 °C shaker for 4 h. Cells were harvested by centrifugation at 5000 *g* for 10 min. Cell pellets were resuspended in 10 ml 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0, and lysed in a French press at 20 000 p.s.i. (138 MPa). Lysates were centrifuged twice at 5000 *g* for 15 min to remove cellular debris and unbroken cells, and the supernatants were centrifuged at 200 000 *g* for 1 h. The membrane pellet was resuspended in 1.0 ml 25 mM Tris/HCl, pH 8.0. For pilus preparation, an overnight culture of UMD916 grown at 37 °C was diluted 1:50 in 500 ml DMEM and grown for 6 h at 37 °C. Cells were harvested by centrifugation at 5000 *g* for 10 min, then resuspended in 100 ml 0.065 M ethanolamine, pH 10 (MEB). The mixture was placed in a Waring 34BL97 commercial blender and sheared for 5 min at setting 4. The resuspension was centrifuged at 4000 *g* for 15 min, the pellet was washed in 50 ml MEB, and the supernatant was stored separately. The pellet and remaining wash were centrifuged again at 4000 *g* for 15 min, and the pellet was stored at -20 °C. This pellet was later lysed for preparation of membrane fractions as described above. The resulting supernatant was combined with the 100 ml MEB sample obtained earlier, then centrifuged once again at 4000 *g* for 15 min. Ammonium sulfate was added to the supernatant to a concentration of 50% (w/v) and allowed to precipitate overnight at 4 °C. The solution was then centrifuged at 27 500 *g* for 15 min and the supernatant discarded. The pellet was resuspended in 30 ml MEB and centrifuged at 21 700 *g* for 30 min. The final pellet was resuspended in 1 ml MEB.

Immunoblotting. Rat polyclonal antisera against purified and refolded His-BfpL was commercially generated (Rockland Immunochemicals) and affinity-purified using a Pierce AminoLink Plus Immobilization kit according to the manufacturer's instructions. This primary antibody was used at a dilution of 1:1000 in commercially available Odyssey Blocking Buffer (LI-COR) and 2% Normal Goat Serum (Sigma). An

anti-rat Alexa Fluor 680-conjugated secondary antibody (Invitrogen) was used at 1:2000. For controls, rabbit antisera against bundlin and BfpB peptides, as previously described (Daniel *et al.*, 2006; Fernandes *et al.*, 2007), were used at a dilution of 1:20 000 in 5% milk + PBS and 1% Tween-20. An anti-rabbit secondary antibody conjugated to an IRDye 800CW fluorophore (LI-COR) was used at a dilution of 1:5000 in Odyssey Blocking Buffer (LI-COR) and 2% Normal Goat Serum (Sigma). All images were taken with an Odyssey Fc Imaging System, with densitometric analysis performed using Odyssey Imager Software (LI-COR).

Dual reporter membrane topology assays. Codons 1–15, 1–35, 1–86, and 1–159 of *bfpL* were amplified by PCR (for primers see Table 2) and cloned into the *XhoI/BamHI* or *AflIII/XhoI* sites of pTEB120 (Blank & Donnenberg, 2001), fusing them to the dual-reporter '*phoA-lacZ*' cassette. The resulting plasmids were transformed into *E. coli* strain XL1-blue and transformants were examined for alkaline phosphatase and β -galactosidase activities by plating on dual-indicator plates (Alexeyev & Winkler, 1999). Plasmids pRPA311 and pRPA312 encoding BfpC_{1–164} and BfpC_{1–188} fused to the dual reporters served as cytoplasmic and periplasmic controls, respectively.

Yeast two-hybrid assays. Transformations of yeast cells were performed using the lithium acetate-mediated method, and quantification of protein–protein interactions used the α -Gal quantification assay as described in the Clontech Yeast Protocols Handbook (Clontech Laboratories, Inc., 2009). Descriptions of each vector used in the quantitative assay are found in Table 1. All absorbance readings from these assays were adjusted to correct for the absorbance of the medium by subtracting the mean A_{410} from eight blank wells containing reaction medium alone from the A_{410} of each culture prior to calculating the α -Gal metabolic units as described in the Clontech Handbook.

Autoaggregation assays. The protocol was adapted from a previous procedure (Anantha *et al.*, 1998). For BFP-inducing conditions, bacterial strains were inoculated in LB with 50 μ g kanamycin ml^{-1} or 20 μ g chloramphenicol ml^{-1} if necessary to retain plasmids, and were grown overnight in a 37 °C shaker at 225 r.p.m. Cultures were diluted 1:50 or 1:200 in 10 ml DMEM with or without 0.2% arabinose, and incubated for 4 h at 220 r.p.m. and 37 °C. Readings were taken each hour in the following manner. One millilitre of culture was removed and its OD₆₀₀ measured with a spectrophotometer. This value was the Before Vortex (BV) reading. The cuvette containing the culture was then vortexed for 30 s and another reading was taken (After Vortex; AV). AV values were adjusted to correct for air bubbles by determining the mean AV value from 10 blank readings of DMEM after vortexing (0.066) and subtracting this value from the AV readings for each culture to yield the Adjusted After Vortex (AAV) value. The autoaggregation index at each time point was determined using the following formula: (AAV–BV)/BV. For micrographs of aggregates, 10 μ l of cells was applied to a glass slide and examined under $\times 100$ magnification. Photomicrographs were taken using a Zeiss AxioCam MRm camera and AxioVision 4.4 software (2005).

Immunofluorescence. The protocol used was modified from a previous procedure (Fernandes *et al.*, 2007). Bacteria were grown under BFP-inducing conditions. Fifty microlitres of culture was placed in a well of a poly-D-lysine eight-well chamber slide and incubated at 37 °C until the culture had evaporated, then the bacteria were fixed on a 65 °C heat block for 15 min. Wells were washed three times with PBS then incubated for 1 h at room temperature with rabbit anti- α_1 -bundlin antiserum at a 1:100 dilution in PBS. Wells were washed three more times, then incubated for 1 h at room temperature with goat anti-rabbit FITC-conjugated antibody (Sigma-Aldrich) diluted 1:300 in PBS. Cells were washed again and the slide

was examined by fluorescence microscopy (excitation 460 nm). Micrographs were taken using Zeiss AxioVision 4.4 software (2005).

TEM. Bacteria were grown under BFP-inducing conditions as above. Ten millilitres of cell culture was subjected to microcentrifugation (2000 g for 15 min) and resuspended in 250 μ l DMEM prewarmed to 37 °C. Ten microlitres of resuspended cells was placed on top of a Formvar-coated 400 mesh copper grid and left to stand for 5 min. The grids were wicked with a small piece of Whatman paper to remove excess solution, then inverted for 15–20 min on a 50 μ l drop of fixative containing 0.1 M Sorensen's phosphate buffer, 2.5% (v/v) glutaraldehyde, 2% (w/v) paraformaldehyde, and 0.18 M sucrose. Grids were wicked again then rinsed twice with drops of water before being placed in 0.25% phosphotungstic acid for 3–5 s, then wicked and dried in the air. An FEI Tecnai T12 transmission electron microscope with FEI Tecnai T12 tomography acquisition automated software was used to examine the grids at a magnification of $\times 11\,000$.

Cell adhesion assay. A HEp-2 cell adhesion assay was performed as described previously (Donnenberg & Nataro, 1995).

Fluorescence polarization. To study the interaction between BfpL and BfpC, the lower-molecular-mass BfpL was labelled with the fluorophore Alexa Fluor 647 using a commercially available kit. Briefly 100 μ l PBS containing BfpL (~140 μ M) was incubated with an amine-reactive form of Alexa Fluor 647 (Invitrogen). After incubation, the labelled product was dialysed three times for 1 h at 4 °C and once overnight at 4 °C in 45 ml PBS with a Slide-A-Lyzer 2 ml MINI dialysis device (Thermo-Scientific) to remove the unreacted dye. Interactions between labelled BfpL and BfpC, also dialysed in PBS, were studied by fluorescence polarization. Fluorescence polarization measurements were recorded using an Aminco-Bowman Series 2 spectrofluorometer (Thermo Fisher Scientific) with motorized Glann–Thompson polarizers in excitation and emission light paths. Alexa Fluor 647 was excited at 630 nm and polarization measured at an emission wavelength of 670 nm. Measured fluorescence polarizations were corrected for different sensitivities to two orthogonal polarizations using an L-format configuration and the G-factor (LeTilly & Royer, 1993; Lundblad *et al.*, 1996).

RESULTS

BfpL is required for BFP expression and associated phenotypes in the context of other Bfp proteins

Wild-type EPEC strain E2348/69 grown in DMEM (conditions favouring BFP expression) forms large aggregates (Fig. 2a). In contrast, a *bfpL* deletion mutant formed no aggregates after 4 h incubation (Fig. 2c). The auto-aggregation phenotype was partially restored with a complementation vector containing the missing gene after induction with 0.2% arabinose (Fig. 2d).

Aggregate formation was quantified by measuring the auto-aggregation index. This index is a measure of the increase in optical density that occurs after aggregates are disrupted by vortexing. After 4 h incubation under BFP-inducing conditions, wild-type EPEC cells and the complemented mutant grown in arabinose had significantly higher auto-aggregation levels than did the *bfpL* mutant or the complemented strain grown in the absence of arabinose (Fig. 2g). However, the aggregation index of the complemented *bfpL* mutant was

Fig. 2. Auto-aggregation of strains 4 h post-inoculation in DMEM and mean auto-aggregation indexes of cells grown in DMEM at 37 °C over time. (a) Wild-type EPEC strain E2348/69; (b) UMD916, the *bfpF* mutant; (c) UMD955, the *bfpL* single deletion mutant; (d) UMD 955 complemented with a plasmid containing *bfpL* and grown in the presence of 0.2% arabinose; (e) UMD961, the *bfpF bfpL* double mutant; (f) UMD961 complemented with a plasmid containing *bfpL* and induced with 0.2% arabinose. Bars, 20 µm. (g) ■, E2348/69, wild-type EPEC; □, UMD955, the *bfpL* deletion mutant; △, UMD955 complemented with a *bfpL* plasmid but with no arabinose added; ◇, UMD955 complemented with a *bfpL* plasmid and induced with 0.2% arabinose. The index of the complemented single mutant was significantly greater ($P < 0.05$) than UMD955 at 4 h, but significantly less than E2348/69 at 4 h ($P < 0.05$). (h) ■, E2348/69, wild-type EPEC; □, UMD916, the *bfpF* mutant; △, UMD955, the *bfpL* deletion mutant; ◇, UMD961, the *bfpF bfpL* double mutant; ×, UMD961 complemented with a *bfpL* plasmid but with no arabinose added; +, UMD961 complemented with a vector containing *bfpL* and induced with 0.2% arabinose. The index of UMD916 is significantly greater than those of all strains at 4 h ($P < 0.05$), while that of the complemented double mutant is significantly greater than UMD955, UMD961 or complemented UMD961 without arabinose at 4 h ($P < 0.05$), and significantly less than UMD916 or E2348/69 at the same time point ($P < 0.05$). (g, h) Error bars, SEM.

significantly lower than that of E2348/69 cells even at 4 h, indicating that complementation was not complete. The decrease in the aggregation index for wild-type EPEC between 3 and 4 h is a result of the high turbidity of the culture prior to vortexing at the 4 h time point compared with the other strains. Failure of complete complementation may be due to expression of BfpL from pLDBADL at levels that differ from those of the wild-type, as more BfpL is expressed in the complemented strain (data not shown).

In a qualitative examination of LA, wild-type EPEC formed typical microcolonies on the surface of HeLa cells (Fig. 3a), while the *bfpL* mutant did not form any microcolonies, adhering as rare single cells (Fig. 3b). Complementation restored the wild-type phenotype (Fig. 3c).

Immunofluorescence microscopy with an antibody against bundlin confirmed that wild-type EPEC cells express

bundlin on the surface. Immunoreactivity was not seen in the *bfpL* mutant (data not shown). In addition, TEM allowed a direct examination of BFP expression. Long, thick, intertwined fibres were found in E2348/69 cells (Fig. 4a) and complemented mutant strains induced with 0.2% arabinose (Fig. 4e), but were not present in the *bfpL* mutant strain without the complementation plasmid (Fig. 4c).

Deletion of the *bfpL* gene in a *bfpF* mutant reveals that *bfpL* is absolutely required for BFP expression

Studies in other T4P systems have revealed that some proteins previously thought to be required for T4P expression are in fact dispensable when pilus retraction is prevented by mutation of the gene encoding the retraction ATPase (Carbonnelle *et al.*, 2006; Morand *et al.*, 2004;

Fig. 3. Adherence of EPEC strains to HeLa cells. Examples of microcolonies are circled. (a) Wild-type EPEC strain E2348/69; (b) the single *bfpL* mutant, UMD955; (c) UMD955 with a *bfpL*-containing plasmid and 0.2% arabinose added as inducer; (d) the *bfpF bfpL* double mutant, UMD961; (e) the *bfpF bfpL* double mutant, UMD961; (f) UMD961 with a *bfpL*-containing plasmid and 0.2% arabinose added as inducer.

Winther-Larsen *et al.*, 2005; Wolfgang *et al.*, 1998). This finding has been interpreted as evidence that the protein in question either facilitates pilus expression or hinders retraction. In EPEC, pilus retraction is mediated by the putative ATPase BfpF (Anantha *et al.*, 1998; Zahavi *et al.*, 2011). While the *bfpF* mutant retained the ability to form aggregates after 4 h incubation in DMEM (Fig. 2b), no aggregates were seen in a *bfpF bfpL* double mutant (Fig. 2e). The *bfpF* phenotype was partially restored when the double mutant was complemented with a plasmid containing *bfpL* and induced with 0.2% arabinose (Fig. 2f). Furthermore, the auto-aggregation index of the complemented *bfpF bfpL* double mutant was slightly but significantly higher than that of the double mutant strain at 4 h (Fig. 2h), although it was still significantly lower than those of wild-type EPEC or the

parent *bfpF* mutant strain. As with the single mutant, complementation was not complete. The *bfpF* mutant formed microcolonies on the surface of HeLa cells (Fig. 3d), but the *bfpF bfpL* double mutant did not display this phenotype (Fig. 3e). Immunofluorescence with the anti-bundlin antibody did not reveal bundlin on the surface of the double mutant. Surface bundlin was detected by immunofluorescence in the *bfpF* mutant and the double mutant complemented with *bfpL* (data not shown). These findings were confirmed using TEM when BFP were detected on a *bfpF* mutant (Fig. 4d), but not on the *bfpF bfpL* double mutant (Fig. 4f). BFP were found only when the double mutant was complemented with *bfpL* (Fig. 4g). Together, these results indicated that BfpL is absolutely required for BFP biogenesis.

Fig. 4. Transmission electron micrographs of EPEC cells grown for 4.5 h in DMEM at 37 °C. Dark, thick, bundled fibres correspond to BFP. Thin, individual fibres may represent the *E. coli* common pilus or another body. (a) Wild-type strain E2348/69; (b) UMD901, a *bfpA* mutant that expresses no BFP; (c) the *bfpL* single mutant UMD955; (d) the *bfpF* mutant UMD916; (e) UMD955 complemented with a plasmid containing *bfpL* and induced with 0.2% arabinose; (f) the *bfpL bfpF* double mutant UMD961; (g) UMD961 complemented with a plasmid containing *bfpL* and induced with 0.2% arabinose.

BfpL localizes to the IM of EPEC

A prior study indicated that BfpL could be detected primarily in the IM fraction of EPEC cells (Ramer *et al.*, 2002). However, the possibility that BfpL is incorporated into BFP was not investigated. Western blots of EPEC lysates separated into soluble fractions (containing cytosolic and periplasmic compartments) and insoluble fractions (containing both membranes and insoluble material) by ultracentrifugation confirmed that BfpL is present exclusively in the insoluble fraction (data not shown). No BfpL could be detected even in a concentrated soluble fraction. Fractionation controls confirmed that BfpB, the outer-membrane secretin protein, was also preferentially localized to the insoluble fraction, while GroEL, a cytoplasmic protein, was found as expected primarily in the soluble fraction. To distinguish between membrane localization and incorporation into BFP, cells were separated into a shear fraction containing BFP and an insoluble membrane fraction from sheared cells. Prior experience indicates that it is difficult to obtain a pure preparation of BFP, and contamination with various non-pilus proteins is expected. However, these preparations are enriched in bundlin (Donnenberg, 2012). Densitometric analysis from four independent experiments revealed a 1:1.65 ratio of BfpL in sheared pilus fractions compared with membrane fractions, similar to the 1:1.34 ratio of BfpB, but distinct from the 2.19:1 ratio of bundlin (Fig. 5). These experiments do not support the hypothesis that BfpL is incorporated into the pilus.

Examination of the amino acid sequence of BfpL predicts a single transmembrane domain, but the absence of predominantly positively charged amino acids on either side of this region (von Heijne, 1986) leaves the topology of the protein ambiguous (Fig. 1). We therefore created a series of plasmids from which fusions of full-length and C-terminally

Fig. 5. BfpL is not enriched in sheared pilus preparations with bundlin. The mean relative intensity of sheared pili fractions divided by membranous fractions from four independent immunoblots for bundlin, BfpB and BfpL. Error bars, SEM. The insert shows a representative Western blot used for densitometric analysis. Equivalent dilutions of membrane (M) and shear preparation (S) samples were loaded onto gels and immunoblotting was performed using antisera raised against the indicated proteins.

truncated derivatives of BfpL with a dual reporter could be expressed. This system relies on the simultaneous expression of the alpha fragment of β -galactosidase and alkaline phosphatase lacking its signal sequence to report cytoplasmic and periplasmic localization, respectively (Alexeyev & Winkler, 1999). Like the periplasmic control plasmid encoding a fusion of BfpC₁₋₁₈₈ to the dual reporters, all BfpL fusions were blue on indicator plates, demonstrating alkaline phosphatase activity. Only the cytoplasmic control plasmid encoding the BfpC₁₋₁₆₄ fusion was red, indicating alpha-complementation with the omega fragment of β -galactosidase and cytoplasmic localization. These data are consistent with an IM topology for BfpL in which only the first five residues are cytoplasmic and most of the protein is localized in the periplasm.

BfpL interacts with the periplasmic C terminus of BfpC

Yeast two-hybrid screens with BfpL and proteins or soluble protein fragments encoded by the *bfp* operon revealed an interaction between BfpL and the periplasmic C terminus of BfpC (BfpC-C), a bitopic IM protein. No interactions were found in screening assays between BfpL and any other Bfp protein or fragment tested, which included bundlin, the N terminus of BfpB, the N terminus of BfpC, BfpD, the N terminus, first and second periplasmic loops of BfpE, BfpG, BfpI, BfpJ and BfpU. The results of a quantitative assay, which included a selection of proteins that were negative on initial screening, were consistent with these results (Fig. 6a). The only interaction that yielded α -galactosidase activity significantly greater than that of the negative controls was between BfpL and BfpC-C; α -galactosidase activities in interactions between BfpL and both the N terminus of BfpC and bundlin were similar to those of the negative controls.

To confirm and quantify the interaction between BfpL and BfpC-C, we used fluorescence polarization, which relies on the faster rotation of individual molecules compared with the same molecules in complex and increased polarization of emitted light. A titration curve can be generated from measurements of polarized fluorescence, and the binding process can be quantitated to predict dissociation constants (LeTilly & Royer, 1993; Lundblad *et al.*, 1996). We measured BfpC concentration-dependent BfpL fluorescence polarization (Fig. 6b) and found that the polarization value increased from 217 millipolarization units (mP) for BfpL–Alexa Fluor 647 to a maximum value of approximately 260 mP after addition of BfpC. While the change in polarization value is moderate, these values are in agreement with polarization models for labelled BfpL (molecular mass ~14 kDa, rotational time ~6 ns, lifetime of Alexa Fluor 647 ~1.9 ns). The fit of a four-parameter logistic curve resulted in the midpoint value of $2.43 \pm 0.64 \mu\text{M}$ with a slope of 0.97 ± 0.23 . A slope close to 1 indicates that the stoichiometry of binding is 1:1 and that the midpoint value represents the dissociation constant. Curves based on

Fig. 6. BfpL interacts with the periplasmic C terminus of BfpC. (a) Quantification of protein–protein interactions in a yeast two-hybrid assay. Interacting proteins are listed along the x axis. Light-grey columns represent interactions between BfpL encoded on the pGADT7 activating domain plasmid and a given partner protein on the pGBKT7 binding domain plasmid, while dark-grey columns indicate the reverse. Data are combined from three experiments; error bars, SEM. Asterisks indicate significant ($P < 0.05$) differences between the indicated interaction and the negative controls, BfpL with null vectors (pGADT7 or pGBKT7 with no inserts) or BfpL with laminin or T-antigen. (b) Fluorescence polarization of BfpL–Alexa Fluor 647 in the presence of various concentrations of the C terminus of BfpC. Excitation was at 630 nm and polarization was measured at an emission wavelength of 670 nm. Points represent the average polarization of two readings at the given concentrations of BfpC added; error bars, SD. The line was fitted to the data points using a logistic function, and the arrow indicates the BfpC concentration for the midpoint of polarization changes.

alternative binding ratios did not fit the data as well. In another set of polarization measurements, we used Alexa Fluor 488 for labelling BfpL and obtained a similar midpoint value of 2.25μ M (data not shown). These measurements confirm that BfpL interacts with BfpC-C.

DISCUSSION

BFP is an essential virulence factor for typical EPEC pathogenesis (Bieber *et al.*, 1998). Thirteen genes encoded by the *bfp* operon are required for pilus biogenesis and

function, while one, *bfpH*, appears to be a pseudogene (Anantha *et al.*, 2000; Ramer *et al.*, 2002). We confirmed that a deletion of the *bfpL* gene, encoding a protein of unknown function that has no known homologues in other T4P systems, results in a loss of pilus expression and associated function. The wild-type phenotype was partially restored when the missing gene was complemented. Thus, *bfpL* expression is required for BFP biogenesis and function in the context of all other Bfp proteins. Since the N terminus of BfpL resembles those of pilin-like proteins BfpI, J and K, we also created a deletion of the *bfpL* gene in a *bfpF* mutant. If BfpL were a pilin-like protein, we would

expect this mutant to be able to express BFP in the absence of the putative retraction ATPase BfpF (Carbonnelle *et al.*, 2006; Giltner *et al.*, 2010; Winther-Larsen *et al.*, 2005). However, contrary to our hypothesis, this double mutant did not express BFP, revealing that BfpL expression is absolutely required for BFP biogenesis. While a conditional requirement for a protein in T4P biogenesis only in the presence of the retraction ATPase is not an exclusive property of pilin-like proteins (Brown *et al.*, 2010), these data indicate that, despite some similarities, BfpL is not functionally similar to pilin-like proteins.

Prior studies have suggested that BfpL is an IM protein, but did not examine pilus shear preparations (Ramer *et al.*, 2002). When we did so, we were unable to obtain evidence that BfpL is incorporated into BFP. Pilin-like proteins have been found as integral components of T4P in *N. meningitidis* (Helaine *et al.*, 2007) and *P. aeruginosa* (Giltner *et al.*, 2010), thus further distinguishing BfpL from pilin-like proteins. Additional analysis using a dual-reporter assay is consistent with a bitopic IM topology for BfpL in which most of the protein protrudes into the periplasmic face. Even when only the first 15 amino acids of BfpL were fused to the dual reporter, alkaline phosphatase activity was detected, indicating that the 11 predicted transmembrane residues are sufficient in conjunction with the linker residues to direct the enzyme to the periplasm.

Based on the interdependence of BfpL and BfpJ and the dependence of BfpI and BfpK on BfpL for their abundance, it had previously been suggested that BfpL might interact directly with these pilin-like proteins. However, we did not obtain evidence of such interactions using a yeast two-hybrid screen. Instead, we were only able to detect an interaction between BfpL and the C-terminal periplasmic face of BfpC, a bitopic component of the IM subassembly complex (Milgotina *et al.*, 2011). Given its IM location and topology, this interaction is entirely plausible. Indeed, we were able to confirm and quantify binding between BfpL and BfpC-C using fluorescence anisotropy. The tentative dissociation constant observed may underestimate the actual affinity between these proteins. We confirmed by circular dichroism that refolded samples of BfpL have secondary structure, as opposed to unfolded samples, which do not (data not shown). However, it is not clear that BfpC-C is entirely folded. The BfpC-C-BfpL interaction and the absolute dependence of BFP biogenesis on BfpL indicate that BfpL is also an essential component of the IM subassembly complex. However, due to the limits of the two-component yeast two-hybrid assay, we cannot exclude interactions between BfpL and other proteins.

It was recently revealed that, despite the absence of sequence similarities, the cytoplasmic domain of BfpC is a homologue of PilM and of the cytoplasmic domain of EpsL (Yamagata *et al.*, 2012). The periplasmic domain of EpsL interacts with EpsM (Sandkvist *et al.*, 2000) and the pilin-like protein EpsG (Gray *et al.*, 2011), while PilM interacts with PilN, PilO and PilP (Ayers *et al.*, 2009). Therefore it is

tempting to speculate that either BfpL, the periplasmic domain of BfpC, or both proteins are homologous to EpsM, PilN, PilO or PilP. EpsM, PilN and PilO share a simple ferredoxin fold (Abendroth *et al.*, 2004b; Sampaleanu *et al.*, 2009). Further structural data will be required to determine whether periplasmic BfpC or BfpL also shares this fold. The lack of sequence similarities has now proven to be poor evidence for refuting homologies among T2S, T4a and T4b systems. Further investigation will also be required to determine whether BfpC or BfpL interacts directly with bundlin.

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REFERENCES

- Abendroth, J., Bagdasarian, M., Sandkvist, M. & Hol, W. G. (2004a). The structure of the cytoplasmic domain of EpsL, an inner membrane component of the type II secretion system of *Vibrio cholerae*: an unusual member of the actin-like ATPase superfamily. *J Mol Biol* **344**, 619–633.
- Abendroth, J., Rice, A. E., McLuskey, K., Bagdasarian, M. & Hol, W. G. (2004b). The crystal structure of the periplasmic domain of the type II secretion system protein EpsM from *Vibrio cholerae*: the simplest version of the ferredoxin fold. *J Mol Biol* **338**, 585–596.
- Abendroth, J., Murphy, P., Sandkvist, M., Bagdasarian, M. & Hol, W. G. (2005). The X-ray structure of the type II secretion system complex formed by the N-terminal domain of EpsE and the cytoplasmic domain of EpsL of *Vibrio cholerae*. *J Mol Biol* **348**, 845–855.
- Alexeyev, M. F. & Winkler, H. H. (1999). Membrane topology of the *Rickettsia prowazekii* ATP/ADP translocase revealed by novel dual *pho-lac* reporters. *J Mol Biol* **285**, 1503–1513.
- Anantha, R. P., Stone, K. D. & Sonnenberg, M. S. (1998). 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.
- Anantha, R. P., Stone, K. D. & Sonnenberg, M. S. (2000). Effects of *bfp* mutations on biogenesis of functional enteropathogenic *Escherichia coli* type IV pili. *J Bacteriol* **182**, 2498–2506.
- Ayers, M., Sampaleanu, L. M., Tammam, S., Koo, J., Harvey, H., Howell, P. L. & Burrows, L. L. (2009). PilM/N/O/P proteins form an inner membrane complex that affects the stability of the *Pseudomonas aeruginosa* type IV pilus secretin. *J Mol Biol* **394**, 128–142.
- 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.
- Blank, T. E. & Sonnenberg, M. S. (2001). Novel topology of BfpE, a cytoplasmic membrane protein required for type IV fimbrial biogenesis in enteropathogenic *Escherichia coli*. *J Bacteriol* **183**, 4435–4450.
- Brown, D. R., Helaine, S., Carbonnelle, E. & Pelicic, V. (2010). Systematic functional analysis reveals that a set of seven genes is

- involved in fine-tuning of the multiple functions mediated by type IV pili in *Neisseria meningitidis*. *Infect Immun* **78**, 3053–3063.
- Carbonnelle, E., Helaine, S., Nassif, X. & Pelicic, V. (2006). A systematic genetic analysis in *Neisseria meningitidis* defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. *Mol Microbiol* **61**, 1510–1522.
- Clontech Laboratories, Inc. (2009). *Yeast Protocols Handbook*. Mountain View, CA: Clontech Laboratories.
- Craig, L., Pique, M. E. & Tainer, J. A. (2004). Type IV pilus structure and bacterial pathogenicity. *Nat Rev Microbiol* **2**, 363–378.
- Crowther, L. J., Anantha, R. P. & Donnenberg, M. S. (2004). The inner membrane subassembly of the enteropathogenic *Escherichia coli* bundle-forming pilus machine. *Mol Microbiol* **52**, 67–69.
- Daniel, A., Singh, A., Crowther, L. J., Fernandes, P. J., Schreiber, W. & Donnenberg, M. S. (2006). Interaction and localization studies of enteropathogenic *Escherichia coli* type IV bundle-forming pilus outer membrane components. *Microbiology* **152**, 2405–2420.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Donnenberg, M. S. (2002). Enteropathogenic *Escherichia coli*. In *Infections of the Gastrointestinal Tract*, 2nd edn, pp. 595–612. Edited by M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg & R. L. Guerrant. Philadelphia, PA: Lippincott Williams & Wilkins.
- Donnenberg, M. S. (2012). The amino terminus of the type IV pilin bundlin is modified by N-methylation. http://figshare.com/articles/THE_AMINO_TERMINUS_OF_THE_TYPE_IV_PILIN_BUNDLIN_IS_MODIFIED_BY_N-METHYLATION/91923.
- 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.
- Fernandes, P. J., Guo, Q. & Donnenberg, M. S. (2007). Functional consequences of sequence variation in bundlin, the enteropathogenic *Escherichia coli* type IV pilin protein. *Infect Immun* **75**, 4687–4696.
- Fröls, S., Ajon, M., Wagner, M., Teichmann, D., Zolghadr, B., Folea, M., Boekema, E. J., Driessen, A. J., Schleper, C. & Albers, S. V. (2008). UV-inducible cellular aggregation of the hyperthermophilic archaeon *Sulfolobus solfataricus* is mediated by pili formation. *Mol Microbiol* **70**, 938–952.
- Giltner, C. L., Habash, M. & Burrows, L. L. (2010). *Pseudomonas aeruginosa* minor pilins are incorporated into type IV pili. *J Mol Biol* **398**, 444–461.
- Gray, M. D., Bagdasarian, M., Hol, W. G. & Sandkvist, M. (2011). In vivo cross-linking of EpsG to EpsL suggests a role for EpsL as an ATPase-pseudopilin coupling protein in the type II secretion system of *Vibrio cholerae*. *Mol Microbiol* **79**, 786–798.
- Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* **177**, 4121–4130.
- Helaine, S., Dyer, D. H., Nassif, X., Pelicic, V. & Forest, K. T. (2007). 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. *Proc Natl Acad Sci U S A* **104**, 15888–15893.
- Karupiah, V. & Derrick, J. P. (2011). Structure of the PilM-PilN inner membrane type IV pilus biogenesis complex from *Thermus thermophilus*. *J Biol Chem* **286**, 24434–24442.
- LeTilly, V. & Royer, C. A. (1993). Fluorescence anisotropy assays implicate protein-protein interactions in regulating *trp* repressor DNA binding. *Biochemistry* **32**, 7753–7758.
- Levine, M. M., Bergquist, E. J., Nalin, D. R., Waterman, D. H., Hornick, R. B., Young, C. R., Sotman, S. & Rowe, B. (1978). *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *Lancet* **311**, 1119–1122.
- Lundblad, J. R., Laurance, M. & Goodman, R. H. (1996). Fluorescence polarization analysis of protein-DNA and protein-protein interactions. *Mol Endocrinol* **10**, 607–612.
- Milgotina, E. I., Lieberman, J. A. & Donnenberg, M. S. (2011). Corrigendum – The inner membrane subassembly of the enteropathogenic *Escherichia coli* bundle-forming pilus machine. *Mol Microbiol* **81**, 1125–1127.
- Morand, P. C., Bille, E., Morelle, S., Eugène, E., Beretti, J. L., Wolfgang, M., Meyer, T. F., Koomey, M. & Nassif, X. (2004). Type IV pilus retraction in pathogenic *Neisseria* is regulated by the PilC proteins. *EMBO J* **23**, 2009–2017.
- Ng, S. Y., Wu, J., Nair, D. B., Logan, S. M., Robotham, A., Tessier, L., Kelly, J. F., Uchida, K., Aizawa, S. & Jarrell, K. F. (2011). Genetic and mass spectrometry analyses of the unusual type IV-like pili of the archaeon *Methanococcus maripaludis*. *J Bacteriol* **193**, 804–814.
- Pelicic, V. (2008). Type IV pili: *e pluribus unum*? *Mol Microbiol* **68**, 827–837.
- Proft, T. & Baker, E. N. (2009). Pili in Gram-negative and Gram-positive bacteria – structure, assembly and their role in disease. *Cell Mol Life Sci* **66**, 613–635.
- Ramboarina, S., Fernandes, P. J., Daniell, S., Islam, S., Simpson, P., Frankel, G., Booy, F., Donnenberg, M. S. & Matthews, S. (2005). Structure of the bundle-forming pilus from enteropathogenic *Escherichia coli*. *J Biol Chem* **280**, 40252–40260.
- 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.
- Ramer, S. W., Schoolnik, G. K., Wu, C. Y., Hwang, J., Schmidt, S. A. & Bieber, D. (2002). The type IV pilus assembly complex: biogenic interactions among the bundle-forming pilus proteins of enteropathogenic *Escherichia coli*. *J Bacteriol* **184**, 3457–3465.
- Saiman, L. & Prince, A. (1993). *Pseudomonas aeruginosa* pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. *J Clin Invest* **92**, 1875–1880.
- Sampaleanu, L. M., Bonanno, J. B., Ayers, M., Koo, J., Tammam, S., Burley, S. K., Almo, S. C., Burrows, L. L. & Howell, P. L. (2009). Periplasmic domains of *Pseudomonas aeruginosa* PilN and PilO form a stable heterodimeric complex. *J Mol Biol* **394**, 143–159.
- Sandkvist, M., Bagdasarian, M., Howard, S. P. & DiRita, V. J. (1995). Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. *EMBO J* **14**, 1664–1673.
- Sandkvist, M., Keith, J. M., Bagdasarian, M. & Howard, S. P. (2000). Two regions of EpsL involved in species-specific protein-protein interactions with EpsE and EpsM of the general secretion pathway in *Vibrio cholerae*. *J Bacteriol* **182**, 742–748.
- Schreiber, W., Stone, K. D., Strong, M. A., DeTolla, L. J., Jr, Hoppert, M. & Donnenberg, M. S. (2002). BfpU, a soluble protein essential for type IV pilus biogenesis in enteropathogenic *Escherichia coli*. *Microbiology* **148**, 2507–2518.
- 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, B. J. & Abu Kwaik, Y. (1998).** Expression of multiple pili by *Legionella pneumophila*: identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. *Infect Immun* **66**, 1768–1775.
- 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 the biogenesis of a type IV pilus. *Mol Microbiol* **20**, 325–337.
- Tacket, C. O., Taylor, R. K., Losonsky, G., Lim, Y., Nataro, J. P., Kaper, J. B. & Levine, M. M. (1998).** Investigation of the roles of toxin-coregulated pili and mannose-sensitive hemagglutinin pili in the pathogenesis of *Vibrio cholerae* O139 infection. *Infect Immun* **66**, 692–695.
- Taha, M. K., Morand, P. C., Pereira, Y., Eugène, E., Giorgini, D., Larribe, M. & Nassif, X. (1998).** Pilus-mediated adhesion of *Neisseria meningitidis*: the essential role of cell contact-dependent transcriptional upregulation of the PilC1 protein. *Mol Microbiol* **28**, 1153–1163.
- Tusnády, G. E. & Simon, I. (2001).** The HMMTOP transmembrane topology prediction server. *Bioinformatics* **17**, 849–850.
- von Heijne, G. (1986).** The distribution of positively charged residues in bacterial inner membrane proteins correlates with the transmembrane topology. *EMBO J* **5**, 3021–3027.
- Winther-Larsen, H. C., Wolfgang, M., Dunham, S., van Putten, J. P., Dorward, D., Løvold, C., Aas, F. E. & Koomey, M. (2005).** A conserved set of pilin-like molecules controls type IV pilus dynamics and organelle-associated functions in *Neisseria gonorrhoeae*. *Mol Microbiol* **56**, 903–917.
- Wolfgang, M., Lauer, P., Park, H. S., Brossay, L., Hébert, J. & Koomey, M. (1998).** PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. *Mol Microbiol* **29**, 321–330.
- Yamagata, A., Milgotina, E., Scanlon, K., Craig, L., Tainer, J. A. & Donnenberg, M. S. (2012).** Structure of an essential type IV pilus biogenesis protein provides insights into pilus and type II secretion systems. *J Mol Biol* **419**, 110–124.
- Zahavi, E. E., Lieberman, J. A., Donnenberg, M. S., Nitzan, M., Baruch, K., Rosenshine, I., Turner, J. R., Melamed-Book, N., Feinstein, N. & other authors (2011).** Bundle-forming pilus retraction enhances enteropathogenic *Escherichia coli* infectivity. *Mol Biol Cell* **22**, 2436–2447.
- 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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