Interaction and localization studies of enteropathogenic
Escherichia coli type IV
bundle-forming pilus outer membrane components

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Typical enteropathogenic Escherichia coli strains express an established virulence factor belonging
to the type IV pili family, called the bundle-forming pili (BFP). BFP are present on the cell
surface as bundled filamentous appendages, and are assembled and retracted by proteins
encoded by the bfp operon. These proteins assemble to form a molecular machine. The BFP
machine may be conceptually divided into three components: the cytoplasmic membrane (CM)
subassembly, which is composed of CM proteins and cytoplasmic nucleotide-binding proteins;
the outer membrane (OM) subassembly and the pilus itself. The authors have previously
characterized the CM subassembly and the pilus. In this study, a more complete characterization
of the OM subassembly was carried out using a combination of biochemical, biophysical and
genetic approaches. It is reported that targeting of BfpG to the OM was influenced by the secretin
BfpB. BfpG and BfpU interacted with the amino terminus of BfpB. BfpU had a complex cellular
distribution pattern and, along with BfpB and BfpG, was part of the OM subassembly.

INTRODUCTION

It is becoming increasingly clear that many cellular processes are carried out, not by individual proteins functioning in
isolation, but by macromolecular complexes and intricate protein networks (Alberts, 1998; Phizicky et al., 2003).
Structural and biochemical characterizations of such macromolecular assemblies are being undertaken to obtain a
better understanding of the cellular processes they control. These considerations also apply to proteins and processes
involved in bacterial pathogenesis. Type IV pili (Tfp) of Gram-negative bacteria are assembled by one such multi-
component molecular machine (Anantha et al., 2000; Ramer et al., 2002). Tfp are filamentous surface appendages that are
expressed by many pathogenic bacteria, including Pseudomonas aeruginosa, Vibrio cholerae, Neisseria gonorrhoeae,
Neisseria meningitidis, Salmonella enterica serovar Typhi, Legionella pneumophila, and enteropathogenic (EPEC) and
enterotoxigenic Escherichia coli (Hobbs & Mattick, 1993; Strom & Lory, 1993; Zhang et al., 2000; Stone & Abu Kwaik,
1998; Taniguchi et al., 1995; Girón et al., 1991). Tfp play a role in diverse processes, such as cellular adhesion (Lee et al., 1994;
Rudel et al., 1995), colonization (Herrington et al., 1988; Tacket et al., 1998), twitching motility (Bradley, 1980;
Henrichsen, 1983; Merz et al., 2000; Wall & Kaiser, 1999), biofilm formation (O’Toole & Kolter, 1998), horizontal
gene transfer (Seifert et al., 1988; Yoshida et al., 1999) and virulence (Tacket et al., 1998; Herrington et al., 1988;
Bieber et al., 1998). Proteins of the Tfp biogenesis machine share extensive sequence similarity to proteins of type II secretion
systems (T2SSs) and DNA uptake systems, and have orthologs among the proteins required for the assembly of
filamentous phages and archaeal flagellae, suggesting that these systems are structurally and mechanistically similar
and share an ancient evolutionary link (Chen & Dubnau, 2003; Russel, 1998; Craig et al., 2004; Sandkvist, 2001;
Peabody et al., 2003).

Tfp are homopolymeric structures composed of the pilin structural protein (Craig et al., 2004). Mature pilin is
generated from the pre-pilin precursor, which contains an unusual short positively charged leader peptide, by a
pre-pilin peptidase, which also N-methylates the nascent amino terminus (Strom et al., 1993). A number of conserved
accessory genes are required for Tfp biogenesis, including those encoding the pre-pilin peptidase, a polytopic cyto-
plasmic membrane (CM) protein, pre-pilin-like proteins, nucleotide-binding proteins, and an outer membrane (OM)
secretin. Mutational analyses have revealed that these

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Abbreviations: AHT, anhydrotetracycline; AI, autoaggregation index;
BFP, bundle-forming pilus; CM, cytoplasmic membrane; DSP, dithio-
propionate; EPEC, enteropathogenic Escherichia coli; pre-pilin
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Received 20 January 2006
Revised 1 May 2006
Accepted 8 May 2006

DOI 10.1099/mic.0.28860-0
proteins are required for functional Tfp biogenesis but not for pre-pilin expression or processing (Alm & Mattick, 1997; Anantha et al., 2000; Ramer et al., 2002). Tfp can be divided into two groups, A and B, based on differences in the signal peptide, cleavage site and genomic organization (Craig et al., 2004). Although the biogenesis of Tfp is not well understood, conditional double-knockout mutants of N. gonorrhoeae have revealed three steps in Tfp biogenesis: pilus processing, pilus formation and pilus extrusion (Wolfgang et al., 1998, 2000). Since the second and third steps appear to be coupled, it is likely that the proteins involved in Tfp biogenesis interact to form a single molecular machine (Hwang et al., 2003; Crowther et al., 2004).

EPEC strains are a leading cause of severe infantile diarrhoea in developing countries (Donnenberg & Kaper, 1992). Typical EPEC strains contain an EPEC adherence factor (EAF) plasmid that is associated with the ability of the bacteria to adhere to the surface of epithelial cells in a distinctive pattern of 3D clusters called localized adherence (Cravioto et al., 1979; Scaletsky et al., 1984; Baldini et al., 1983). In liquid culture, the cells form dynamic bacterial aggregates; phenomena called autoaggregation and disaggregation (Vuopio-Varkila & Schoolnik, 1991; Kaper et al., 1992). Both these phenotypes are dependent on a Tfp called the bundle-forming pilus (BFP), an established EPEC virulence factor (Kaper et al., 1993). BFP is encoded by the second gene of the bfp operon (Crowther et al., 2004), and DsbA, which catalyses formation of a disulfide linkage in the periplasmic carboxyl-terminus of bundlin (Zhang & Donnenberg, 1996). A kinetic analysis of bundlin maturation has revealed that during processing, pre-bundlin exists as a CM protein accessible to both enzymes simultaneously (Donnenberg et al., 1992; Sohle et al., 1993). Processing of pre-bundlin to bundlin is dependent on two enzymes: pre-pilin peptidase, a bfpP gene product that cleaves the cytoplasmic signal sequence (Zhang et al., 1994), and DsbA, which catalyses formation of a disulfide linkage in the periplasmic carboxyl-terminus of bundlin (Zhang & Donnenberg, 1996). A kinetic analysis of bundlin maturation has revealed that during processing, pre-bundlin exists as a CM protein accessible to both enzymes simultaneously (Donnenberg et al., 1997). BfpP also processes the pre-pilin-like proteins encoded by the bfpI, bfpJ and bfpK genes (Ramer et al., 2002). BfpB, the product of the bfpA gene, belongs to the secretin family of proteins. BfpB is a lipoprotein and forms multimers that may have pore-forming activity (Ramer et al., 1996). BfpG is encoded by the second gene of the bfp operon and has no homologues. Immunoprecipitation and cross-linking studies have shown that BfpG interacts with BfpB (Schmidt et al., 2001; Hwang et al., 2003). BfpU is a small protein encoded by the bfpU gene, which localizes to both the cytoplasm and periplasm. Its function is not yet understood and it has no known homologues (Schreiber et al., 2002). The bfpD gene encodes a hexameric cytoplasmic ATPase, while the bfpF gene encodes a putative cytoplasmic nucleotide-binding protein involved in pilus retraction (Anantha et al., 1998; Bieber et al., 1998). BfpE is a polytopic CM protein (Blank & Donnenberg, 2001) and BfpC is a bitopic CM protein (Crowther et al., 2004). In the presence of the cytoplasmic amino terminus of BfpC and a peptide derived from the cytoplasmic amino terminus of BfpE, BfpD is a powerful ATPase (Crowther et al., 2005). A single hexamer of BfpD is capable of hydrolysing 455 molecules of ATP per second.

Our group recently provided evidence for a CM subassembly of the BFP biogenesis machine (Crowther et al., 2004). This subassembly contains the bitopic CM protein BfpC, polytopic CM protein BfpE, as well as the cytoplasmic ATPase BfpD and the putative ATPase BfpF. The amino termini of BfpC and BfpE interact with each other and with the BfpD ATPase. These interactions induce conformational changes in each protein, and are required for bacterial autoaggregation and hence for pilus biogenesis. In contrast, BfpF interacts only with the small (25 aa) cytoplasmic loop of BfpE, and this interaction is required for disaggregation and hence pilus retraction.

Hwang et al. (2003) have demonstrated that the OM protein BfpB can be cross-linked with nine of the 13 other BFP proteins. Only BfpH, which may not be expressed, the pre-pilin peptidase BfpP, and the pre-pilin-like proteins BfpJ and BfpK, could not be detected in the cross-linked complexes. Since the BfpB cross-linked complex contained both CM components, such as BfpC and BfpE, and OM components BfpB and BfpG, an oligomeric complex spanning the periplasmic space was proposed. Schmidt et al. (2001) have demonstrated that BfpB and BfpG are present in the OM, and have provided evidence that BfpG is required for formation and/or stability of the BfpB multimer. Based on these observations, it has been proposed that BfpB and BfpG form the OM component of the BFP machine. As a conceptual framework to facilitate experimentation, we envision the BFP machine to consist of a CM subassembly and an OM subassembly. Since BfpU is present in the periplasm in addition to the cytoplasm (Schreiber et al., 2002), our hypothesis states that BfpU interacts with BfpB and BfpG as part of the OM subassembly. As a corollary to this hypothesis, we predicted that the absence of some proteins of the OM subassembly would alter or influence the targeting of the interacting partners within the cell. In this study, we investigated interactions among BfpB, BfpG and BfpU in order to characterize the OM subassembly of the BFP biogenesis machine.

METHODS

**E. coli** strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. SlyD is a histidine-rich peptidyl-prolyl trans-isomerase that can contaminate proteins purified by metal chelation chromatography (Hottenrott et al., 1997). To construct a slyD mutant to facilitate protein purification, we used a one-step lambda Red recombine-facilitated mutagenesis protocol to delete the slyD gene of *E. coli* strain BW25113, replacing it with the sequences of a chloramphenicol-resistance gene (Datsenko &
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description/genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5x</td>
<td>deoR endA1 gyrA96 hisD17 recA1 relA1 supE44 thi-1</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>BW25113</td>
<td>lacI46 proA2 ΔlacZYA::argF U169 (w80lacZM15)</td>
<td>Datsenko &amp; Wanner (2000)</td>
</tr>
<tr>
<td>BL21(AI) slyD</td>
<td>F−ompT hsdRI (F− ρ857 gmr dcm arahB Δ78Rnap−tetA ΔslyD::cat)</td>
<td>This study</td>
</tr>
<tr>
<td>E2348/69</td>
<td>Serotype O127:H6 EPEC strain isolated from an outbreak in the UK</td>
<td>Levine et al. (1978)</td>
</tr>
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<td>UMD922</td>
<td>E2348/69 ΔbfpU::apha-3</td>
<td>Schreiber et al. (2002)</td>
</tr>
<tr>
<td>UMD923</td>
<td>E2348/69 ΔbfpB::apha-3</td>
<td>Anantha et al. (2000)</td>
</tr>
<tr>
<td>UMD928</td>
<td>E2348/69 ΔbfpG::apha-3</td>
<td>Anantha et al. (2000)</td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hisD17 supE44 relA1 lac [F−proAB lacIqZM15 Tn10 (TetR)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>RY3080</td>
<td>BL21(DE3) slyD7</td>
<td>Roof et al. (1997)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pASK-IBA3</td>
<td>Strep-tag expression vector</td>
<td>IBA</td>
</tr>
<tr>
<td>pBAD24</td>
<td>AmpR, L-arabinose-inducible expression vector</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pCRT7/CT-TOPO</td>
<td>ColE ori, AmpR, ZeoR, T7 promoter, T-A cloning, V5 epitope, His6</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pGADT7</td>
<td>Yeast two-hybrid vector containing GAL4 activation domain</td>
<td>BD Biosciences–Clontech</td>
</tr>
<tr>
<td>pGBK7</td>
<td>Yeast two-hybrid vector containing GAL4 DNA binding domain</td>
<td>BD Biosciences–Clontech</td>
</tr>
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<td>pMSD235</td>
<td>XbaI–SacII fragment of pMSD234 containing bfpU with additional six His codons at the 3’ end cloned in pRPA103</td>
<td>Schreiber et al. (2002)</td>
</tr>
<tr>
<td>pTEB68</td>
<td>bfpE–His expression vector</td>
<td>McNamara et al. (2001)</td>
</tr>
<tr>
<td>pWS16</td>
<td>bfpG–His gene cloned into pTEB68</td>
<td>This study</td>
</tr>
<tr>
<td>pWS15</td>
<td>bfpB–Strep gene cloned into pASK-IBA3</td>
<td>This study</td>
</tr>
<tr>
<td>pWS52</td>
<td>bfpG–C9S, C14S–His gene cloned into pTEB68</td>
<td>This study</td>
</tr>
<tr>
<td>pAD07</td>
<td>bfpU–His gene cloned into pBAD24</td>
<td>This study</td>
</tr>
<tr>
<td>pAD017</td>
<td>Sequences of bfpB encoding amino acids 19–171 cloned into pCRT7/CT-TOPO vector</td>
<td>This study</td>
</tr>
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</table>

Bacterial strains were cultured in Luria–Bertani broth at 37 °C. BFP was expressed as previously described (Schreiber et al., 2002). Antibiotics were added at the following concentrations to select for or maintain plasmids: ampicillin, 100 μg ml−1; chloramphenicol, 20 μg ml−1; kanamycin, 50 μg ml−1.

**Autoaggregation assays.** The autoaggregation phenotype, which requires expression of BFP (Anantha et al., 2000), was tested as previously described (Crowther et al., 2004). The autoaggregation index (AI), which measures the percentage increase in optical density following disruption of aggregates after vortexing as a function of time, was also determined as described by Anantha et al. (1998).

**Chemical cross-linking and affinity purification of complexes.** Overnight cultures of *E. coli* UMD922(pMSD235) expressing BfpU tagged at its carboxyl terminus with hexahistidine (His6) (BfpU–His) in a bfpU mutant background (Schreiber et al., 2002); strain UMD928(pWS16) expressing His6-tagged BfpG (BfpG–His) in a bfpG mutant background; and strain UMD923(pWS15) expressing Strep-tagged BfpB (BfpB–Strep) in a bfpB mutant background, were grown under BFP-inducing conditions. For strain UMD928 (pWS16), IPTG (1 mM final concentration) was added to the cultures to induce BfpG expression, and for strain UMD923(pWS15), anhydrotetracycline (AHT) was added (20 μg 100 ml−1 final concentration) to induce BfpB expression. Cells were harvested, washed and resuspended in PBS to OD600 1.0. The membrane-permeable, homobifunctional thiol-cleavable, chemical cross-linker dithiobis-[succinimidyl]propionate (DSP) (Pierce) was added to a final concentration of 0.125 mM, and incubated at room temperature for 30 min. The reaction was quenched with 0.25 vol. 1 M Tris/HCl (pH 7.4) for 15 min at room temperature. For strains UMD928 (pWS16) and UMD922(pMSD235), cells were resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed in a French press at 20 000 p.s.i. (~138 000 kPa). The lysates were cleared by centrifugation and the cross-linked complexes were purified by nickel-nitrirotriacetic acid (Ni-NTA) (Qiagen) affinity chromatography according to the manufacturer’s instructions. For strain UMD923(pWS15), cells were resuspended in buffer A (100 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, pH 7.0) and lysed in a French press at 20 000 p.s.i. (~138 000 kPa). The lysates were centrifuged at 6000 g to remove cell debris, and membranes were pelleted at 100 000 g. The membrane preparation was solubilized in buffer A + 2 % SDS for 10 min, and unsolubilized material was removed by centrifugation at 100 000 g. The SDS concentration in the supernatant was decreased to <0.1 % with buffer A. Complexes were purified using affinity chromatography by mixing with a slurry of Strep-Tactin Sepharose (IBA) for 20 min at room temperature. The slurry was loaded on a column and washed twice with buffer A, followed by elution with buffer B (buffer A + 2.5 mM desthiobiotin). Eluted proteins were dialysed against buffer A to remove desthiobiotin and concentrated by ultrafiltration (Centricon
PL-10; Millipore). Flow-through and eluates of all complexes were concentrated and analysed by SDS-PAGE electrophoresis in the presence or absence of β-mercaptoethanol, (β-ME) or by Western blotting.

**Preparation of soluble and insoluble (membrane) fractions.** Soluble and insoluble membrane fractions were prepared as previously described by O’Connell et al. (2004). The protein concentrations of the soluble and insoluble fractions were measured with the bicinchoninic acid method (Pierce), and NADH oxidase assays were performed as previously described by Osborn et al. (1972).

**Western blotting.** Western blotting was performed as described previously (Schreiber et al., 2002). Primary antibodies were used at the following dilutions: monoclonal anti-bundlin ICA4, 1:30 000 (Giron et al., 1995); monoclonal anti-BfpU, 1 : 30 000 (Schreiber et al., 2002); anti-maltose-binding protein (MBP) 1 : 10 000 (New England Biolabs); anti-GroEL conjugated to horseradish peroxidase (HRP) 1 : 80 000 (Sigma). A rabbit polyclonal antiserum was raised against purified BfpC1–164 (Crowther et al., 2004) and used at a dilution of 1 : 30 000. A rabbit polyclonal antibody was raised against purified BfpG–His, absorbed thrice against an acetone powder of bfpG mutant strain UMD928, and used at a dilution of 1 : 10 000. The polyclonal BfpB antiserum was obtained commercially (Research Genetics) from rabbits immunized with a multiple-antigenic peptide (NILHADTLSKSNKHE–YKJSSD) representing amino acids 34–55 of BfpB, and used at a dilution of 1 : 15 000. Secondary goat antimouse IgG or goat anti-rabbit IgG antiserum conjugated to HRP (Amersham Pharmacia Biotech) were used at a dilution of 1 : 30 000. Blots were developed by enhanced chemiluminescence using the ECL kit (Amersham Pharmacia Biotech). When necessary, blots were stripped using Stripping Buffer (Pierce), according to the manufacturer’s instructions.

**Sucrose density gradient fractionation.** Membrane fractionation using sucrose flotation density centrifugation was performed as described previously (Anantha et al., 2000). The presence of OmpA as a marker of OM protein fractions was assessed by Ponceau staining as described by Anantha et al. (2000).

**Periplasmic fraction preparation.** A method employing gentle Triton X-100 detergent treatment to separate periplasmic and cellular fractions of bacteria was used as previously described (Schreiber et al., 2002).

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**Table 2. Primers used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donne-402</td>
<td>5’-CAT GCC ATG GTG AGG ACA GTA ATC CTT TTT T-3’</td>
<td>Forward primer for BfpG purification</td>
</tr>
<tr>
<td>Donne-405</td>
<td>5’-CGG GAT CCT CCT TCG GTG GAT TGT GTA AC-3’</td>
<td>Reverse primer for BfpG purification</td>
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<tr>
<td>Donne-419</td>
<td>5’-CAA GAT GGT CTC AGC GCT TTC GCC ATA AGC CCT GAG AT-3’</td>
<td>Reverse primer for BfpB purification</td>
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<tr>
<td>Donne-420</td>
<td>5’-AAG GAT GGT CTC AAA TGA AAC TTG GCA GGT ATT C-3’</td>
<td>Forward primer for BfpB purification</td>
</tr>
<tr>
<td>Donne-503</td>
<td>5’-CAT GCC ATG GTG AGG ACA GTA ATC CTT TTT TTGA GTC TTT GAG TTT CCA GAT TCA CAA AGC CCA CGA CAT TAT TCA GGC AAA AAA AAG GGG CAG ATG AAT AAT CTT TTT CTA TGC CTA GGA GAT ATC GTG TAG GCT GGA GCT GTC T-3’</td>
<td>Forward primer for BfpB purification</td>
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<tr>
<td>Donne-733</td>
<td>5’-ATC GGC TGG CAG GCT GAA GAA ACG CCA CCG CCA CAT TAT TGA GGC GTA AAA AAA AAG GGG CAG ATG AAT AAT CTT TTT CTA TGC CTA GGA GAT ATC GTG TAG GCT GGA GCT GTC T-3’</td>
<td>Forward primer for slyD deletion</td>
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<tr>
<td>Donne-74</td>
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<td>Donne-919</td>
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<td>Donne-968</td>
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<td>5’ End of bfpB55-513 in pCRT/CT-TOPO</td>
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<td>Donne-969</td>
<td>5’-AAG TAC AGG TTT GGA GTT TCC GCC CGC-3’</td>
<td>3’ End of bfpB55-513 in pCRT/CT-TOPO</td>
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</table>
Purification of carboxyl-terminal His_6-tagged bfpU. The bfpU gene, including the codons for a carboxyl-terminal His_6 tag, was PCR-amplified from pMSD235 (Schreiber et al., 2002) with primers Donne-874 and Donne-875 (Table 2), cloned into the Ncol–SalI sites of pBAD24 (Guzman et al., 1995), and confirmed by sequencing. The resulting plasmid, named pAD07, was electroeluted into DH5α and BL21 (AI) slyD strains. For overexpression of BfpU–His, strain BL21 (AI) slyD (pAD07) was grown in MOPS Minimal Medium to prevent formation of inclusion bodies. Cultures were induced at OD_600 ~ 0.3 with 1 mM IPTG overnight. Cells were lysed in a French press in lysis buffer. BfpU–His was batch-purified by Ni-NTA affinity chromatography (Qiagen), further purified with a nickel HiTrap HP column (Amersham Biosciences) according to the manufacturer’s instructions, and purity-assessed by SDS-PAGE and silver staining.

Purification of BfpB and BfpG. The bfpG gene was amplified with primers Donne-402 and Donne-405 (Table 2), and the resulting PCR product was cloned in pTEB68 (Table 1) so as to replace the bfpE gene with bfpG, creating a gene fusion with the codons for six histidine residues at the 3’ end. The resulting plasmid was designated pWS16. Alternatively, the bfpG gene was amplified with primers Donne-303 and Donne-405 (Table 2) to replace both codons for cysteine 9 and cysteine 14 with serine, and cloned into pTEB68 (Table 1) to create pWS52. BfpG–His was purified either from the complemented bfpG mutant strain UMD928 (pWS16) or E. coli RY3080 (pWS16) by Ni-NTA affinity chromatography (Qiagen), according to the manufacturer’s instructions. The purified protein from the complemented bfpG mutant was analysed by silver staining after electrophoresis through an 8–16% gradient polyacrylamide gel, and transferred to a PVDF membrane for Edman degradation. The resulting plasmid, named pAD07, was electroporated into BL21 (AI) slyD strain. BfpB19–171 was refolded by sequential dialysis against the latter buffer with 3, 1 and 0 M urea. The resulting plasmid was amplified with primers Donne-402 and Donne-405 (Table 2), and the resulting PCR product was cloned in pTEB68 (Table 1) to create pWS52. BfpG–His was purified either from the complemented bfpG mutant strain UMD928 (pWS16) or E. coli RY3080 (pWS16) by Ni-NTA affinity chromatography (Qiagen), according to the manufacturer’s instructions. The purified protein from the complemented bfpG mutant was analysed by silver staining after electrophoresis through an 8–16% gradient polyacrylamide gel, and transferred to a PVDF membrane for Edman degradation sequencing by the University of Virginia Biomolecular Research Facility.

To purify BfpB, the bfpB gene was amplified using primers Donne-420 and Donne-419 (Table 2), and cloned into pASK-IBA3 to add a carboxyl-terminal Strep-tag (Table 1). BfpB–Strep was purified from XL-1 Blue (pWS15) by Strep-Tactin Sepharose affinity chromatography, as described for purification of BfpB-cross-linked complexes.

Purification of the His_6-tagged amino-terminal third of bfpB. The fragment of bfpB encoding amino acids serine 19 to glycine 171, referred to hereafter as BfpB<sub>19–171</sub>, was PCR-amplified with primers Donne-968 and Donne-969 (Table 2), and cloned into pCRT7/CT-TOPO (Invitrogen), which adds a carboxyl-terminal His<sub>6</sub> tag. The resulting plasmid, named pAD017, was electroeluted into the BL21 (AI) slyD strain. BfpB<sub>19–171</sub> was purified under denaturing conditions with Ni-NTA (Qiagen) affinity chromatography according to the manufacturer’s instructions. The purified protein was concentrated by ultrafiltration (Centricon PL-10; Millipore).

Yeast two-hybrid analysis. Yeast two-hybrid analysis was done using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech) according to the manufacturer’s instructions. Primers designed to amplify sequences of bfpB encoding BfpG lacking its signal sequence, bfpB encoding BfpB lacking its signal sequence, BfpB<sub>19–171</sub> and carboxyl-terminal BfpB (Ala172 to Glu553), referred to hereafter as BfpB<sub>225–553</sub>, were used to generate fragments subsequently cloned into vectors pGBK7T and pGADT7 (Table 2).

Microcalorimetry titration studies. Isothermal titration calorimetry (ITC) measurements were carried out at 37°C with a VP-ITC Microcalorimeter (MicroCal). All titrations were performed in 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 7.0. In each ITC experiment, 3 μl aliquots of 0–12 mM BfpB (amino-terminal third) were injected from a 250 μl rotating (280 r.p.m.) syringe into the 1:4 ml isothermal sample chamber containing 0.003 mM BfpU. Experiments consisted of 35 injections of 6 s duration at 210 s intervals. In corresponding control experiments, the amino-terminal third of BfpB was injected into buffer alone. These heats of dilution of BfpB were subtracted from the heats generated in the titrations with BfpU. Calorimetric data analysis was carried out with ORIGIN 5.0 software (MicroCal). Binding parameters, such as the stoichiometry of binding (n), the binding constant (K<sub>b</sub>), and the binding enthalpy (ΔH<sub>b</sub>), were determined by fitting the experimental binding isotherm. Three ITC experiments were performed and the mean binding parameters and associated errors were calculated.

RESULTS

Purification and analysis of BfpG

The BfpG protein is encoded by the second gene in the bfp operon. We had previously suggested that BfpG might be a lipoprotein, based on a possible signal peptidase II cleavage site before cysteine 14 of the putative pre-protein (Stone et al., 1996). To test this hypothesis, we first cloned the bfpG gene into an expression vector that would allow purification of BfpG on the basis of a carboxyl-terminal His<sub>6</sub> tag. The resulting plasmid, pWS16, was introduced into the bfpG mutant strain UMD928, and transformants were examined after growth in BFP-expressing conditions in the presence of various concentrations of IPTG to induce expression of BfpG–His. We found typical large autoaggregates in cultures grown for ≥ 4 h in the presence of ≥ 0.01 mM IPTG. In contrast, no autoaggregates were seen in cultures of the bfpG mutant grown under similar conditions. To quantify the effect, we calculated the AI of the bfpG mutant and the complemented bfpG mutant (Fig. 1A). Compared to the bfpG mutant, both wild-type EPEC E2348/69 and the complemented bfpG mutant UMD928 (pWS16) displayed a steady increase in AI. This result indicates that the addition of a carboxyl-terminal His<sub>6</sub> tag did not interfere with the function of BfpG. We used PCR to replace with serine the codons for both cysteine 9 and cysteine 14, the latter predicted to be essential for signal peptidase II cleavage and modification of pre-BfpG. The resulting plasmid, pWS52, was identical to pWS16 except for this double mutation and, like pWS16, it was able to complement strain UMD928 for autoaggregation, with increasing AI similar to levels seen when the mutant was complemented with the wild-type gene (Fig. 1A). This result suggests that signal peptidase II cleavage is not required for BfpG function. We confirmed the expression of BfpG by Western blotting, and found that BfpG was expressed only in the presence of IPTG in strains UMD928 (pWS16) and UMD928 (pWS52) (Fig. 1B). The migration of the BfpG–His proteins was slower than that of BfpG, as expected.

We expressed BfpG–His in strain UMD928 and purified the protein by Ni-NTA chromatography. The purified protein migrated on gels as a doublet. The M<sub>r</sub> of the slower migrating band was 14 700 and that of the faster migrating band was 13 800. Amino-terminal sequencing of the slower band revealed the following amino acids: QESANKNEKLFS.
while no autoaggregates were seen in the presence of AHT at concentrations <0.02 μg ml⁻¹. We conclude that the addition of a carboxyl-terminal Strep peptide tag does not impair the function of BfpB. BfpB–Strep purified from EPEC, as well as E. coli K-12 backgrounds, was also analysed by electron microscopy (data not shown). We observed ring-shaped structures with outer and inner diameters similar to those previously reported (Schmidt et al., 2001).

**BfpB, BfpU and BfpG influence membrane targeting of each other**

Previous studies on localization of various BFP proteins have indicated that BfpG localizes to the OM along with BfpB (Ramer et al., 1996; Schmidt et al., 2001), while BfpU localizes to the cytoplasm and periplasm (Schreiber et al., 2002). Since these proteins are present in the OM–periplasm–CM interface, they could potentially interact with one another in the BFP machine and, in turn, influence the localization of each protein to particular cellular compartments. Therefore, we investigated whether the absence of BfpU, BfpG or BfpB proteins altered the localization of each protein. For this analysis, wild-type, mutants and mutant-complemented EPEC strains, as well as laboratory E. coli strains expressing only the BFP protein of interest, were fractionated into insoluble (representing the OM and CM proteins) and soluble (representing the cytoplasm and periplasm proteins) fractions. Periplasmic fractions were also prepared separately. Western blotting was conducted on these fractions with specific antibodies. The purity of each fraction was determined by Western blotting with anti-GroEL (a cytoplasmic resident protein) and NADH oxidase (a CM protein) assays. The results of the Western blotting are shown in Fig. 2. The distribution of NADH oxidase and GroEL revealed little or no contamination of the insoluble/soluble fractions. BfpB was detected predominantly in the insoluble fraction of wild-type EPEC strain E2348/69, the bfpU mutant UMD922 and the bfpG mutant UMD928, as well as in the insoluble fraction of laboratory E. coli strain XL-1 Blue(pWS15) expressing BfpB–Strep, indicating that targeting of BfpB to the membrane was independent of BfpU, BfpG, or any other BFP protein (Fig. 2, first panel). BfpU was present predominantly in the soluble fractions of wild-type EPEC, bfpG mutant UMD928 and the bfpB mutant UMD923 (Fig. 2, second panel). Although less abundant, BfpU was also detected in the insoluble fractions of wild-type EPEC and the bfpG and bfpB mutants. These results indicate that BfpU may be present not only in the cytoplasm and periplasm, as previously reported (Schreiber et al., 2002), but might also be associated with the membrane. BfpG was detected in the insoluble and soluble fractions of wild-type EPEC and the bfpU mutant (Fig. 2, third panel). However, in the bfpB mutant, BfpG was detected only in the soluble fraction. In the laboratory E. coli strain RY3080(pWS16) expressing only BfpG–His, BfpG was detected predominantly in the soluble fraction. The small amount of BfpG detected in the insoluble fraction of strain RY3080(pWS16) may have been due to overexpression of

Fig. 1. BfpG cysteine residues 9 and 14 are not required for function. Overnight cultures of wild-type strain E2348/69 (●), bfpG mutant strain UMD928 (□), UMD928 complemented with pWS16 encoding wild-type BfpG (▲), and UMD928 complemented with pWS52 encoding BfpG<sub>C298S C14S</sub> (○), were diluted 1:250 in DMEM and incubated at 37°C. (A) AIs were determined on samples at 2, 3, 4 and 5 h. Bars indicate SEM of three independent experiments. (B) Five-hour samples grown in the presence or absence of 0·1 mM IPTG, as indicated, were analysed by immunoblotting with anti-BfpG antibody. WT, wild-type.

**Purification of BfpB–Strep**

To aid in the purification of BfpB, we expressed the protein in pWS15, which encodes BfpB–Strep. To ensure that the modified protein was functional, pWS15 was introduced into bfpB mutant strain UMD923 and assayed for autoaggregation. Strain UMD923(pWS15) formed typical large autoaggregates similar to those formed by wild-type EPEC under BFP-inducing conditions in the presence of AHT, sequence is a perfect match for residues 19–30 of BfpG and follows a consensus signal peptidase I cleavage site. The faster migrating band revealed the sequence ALQ(A)SERTI(K)NA, which matches amino acids 37–48, and also follows a consensus signal peptidase I cleavage site. Thus, we conclude that mature BfpG is not a lipoprotein, but rather is processed from pre-BfpG at two alternative sites by signal peptidase I.
BfpG producing some insoluble protein. These results suggest that targeting of BfpG to the membrane is dependent on the presence of BfpB.

Since localization of BfpG to the membrane appeared to be dependent on the presence of BfpB, and BfpG contained a cleavable signal peptide, we hypothesized that BfpG should have been present in the periplasmic fraction of the bfpB mutant. To test this hypothesis, periplasmic fractions were prepared by treating the cells gently with detergent, followed by Western blotting with anti-BfpG antibodies. BfpG was detected in approximately equal proportions in the periplasmic and cellular pellet fractions of wild-type EPEC (Fig. 3, top panel). However, in the bfpB mutant, BfpG was detected predominantly in the periplasmic fraction. When the bfpB mutant was complemented with a plasmid encoding BfpB–Strep protein, the distribution profile of BfpG was similar to wild-type EPEC, indicating that in the absence of BfpB, BfpG localizes primarily in the periplasm.

Western blotting of the periplasmic and cellular pellet fractions of wild-type EPEC with anti-BfpU antibodies detected BfpU in both fractions, indicating that only a portion of the BfpU molecules was present in the periplasm. In contrast, in the bfpB mutant, BfpU was detected primarily in the periplasmic fraction, while only a small amount was detected in the cellular pellet fraction. Complementation of the bfpB mutant resulted in an increase in the amount of BfpU found in the cellular pellet fraction (Fig. 3, second panel). Thus, in the absence of BfpB, there was a redistribution of BfpU away from the soluble periplasmic fraction. Control experiments with anti-GroEL and anti-MBP antibodies demonstrated the relative purity of the fractions.

**BfpB influences membrane targeting of BfpG and BfpU**

Fractionation of cellular components into insoluble/soluble or periplasmic fractions indicated that BfpB, BfpG and BfpU were targeted to the membrane. To determine whether BfpB, BfpU or BfpG influence the sorting of each other to the CM or OM, we subjected various strains to sucrose density flotation gradients and Western blot analysis (Fig. 4). Successive aliquots removed from the top to the bottom of gradients from wild-type EPEC and the bfpG and bfpU mutants, as well as a laboratory E. coli strain expressing BfpB–Strep, were probed with anti-BfpB antibodies, as

![Fig. 2. Influence of BfpB, BfpU and BfpG on membrane targeting of each protein.](http://mic.sgmjournals.org)

Cultures of indicated strains were separated into insoluble (I) and soluble (S) fractions as described in Methods. Samples were analysed by Western blotting with the indicated antibodies. Purity of sample preparations was assessed by Western blotting using an anti-GroEL antibody and by measuring NADH oxidase activity, shown as a percentage of the total present in each pair of samples. The lanes are labelled as follows: WT, wild-type EPEC strain E2348/69; bfpG, bfpG mutant UMD928; bfpU, bfpU mutant UMD922; bfpB, bfpB mutant UMD923; bfpB + pBfpB, bfpB mutant complemented with pWS15; pBfpB, E. coli strain XL-1 Blue(pWS15); pBfpG, E. coli strain RY3080(pWS16).
Fig. 4. OM targeting of BfpG is dependent on BfpB. Lysates of the indicated strains were applied to the bottom of 32–60 % sucrose gradients and centrifuged at 288 000 g. Aliquots were removed from the top of the gradient, weighed, assayed for NADH activity and analysed by Western blotting. (A) Graphs showing the NADH oxidase activity for each fraction as a percentage of the total NADH oxidase activity (right y axis) and the density of each fraction (left y axis). Western blotting was with anti-BfpB antibodies (B), anti-BfpG antibodies (C) and anti-BfpU antibodies (D). The strains tested are labelled according to Fig. 2.
shown in Fig. 4(B). BfpB was detected primarily in the fractions corresponding to the OM, as well as in the bottom of the gradient (containing soluble and insoluble proteins) in wild-type EPEC. The distribution of BfpB in the bfpG or bfpU mutant was similar to that of wild-type EPEC. In the E. coli K-12 strain expressing BfpB–Strep alone, the distribution of BfpB was broader, but peaked in the OM fractions. This result was probably due to overexpression of BfpB in this strain. These results indicate that BfpB was targeted to the OM and the targeting did not depend on the presence of either BfpG or BfpU.

Gradients containing proteins from wild-type EPEC, the bfpB mutant, the complemented bfpB mutant and the bfpU mutant were probed with anti-BfpG antibodies (Fig. 4C). BfpG was detected primarily in the fractions corresponding to the OM of wild-type EPEC, similar to BfpB. In the bfpU mutant, BfpG was detected only in the fractions corresponding to the OM. However, in the bfpB mutant, BfpG was detected only in the pellet fraction of the gradient containing soluble (cytoplasm/periplasm) and insoluble proteins. In contrast, in the complemented bfpB mutant, BfpG was present in the OM fractions, similar to wild-type EPEC, in addition to the pellet fraction. These results correlate well with the observations of insoluble/soluble and periplasmic protein separation seen above, and indicate that BfpB is required for targeting of BfpG to the OM.

The results of probing gradient fractions from wild-type EPEC, the bfpG mutant, the bfpB mutant and the complemented bfpB mutant with anti-BfpU antibodies are shown in Fig. 4(D). BfpU was detected in fractions corresponding to the OM, as well as in the pellet fractions, indicating that BfpU seen in the insoluble fraction of wild-type EPEC (Fig. 2, second panel) was due to its association with the OM. The distribution of BfpU in the gradients from the bfpG and bfpB mutants appeared to differ from that in the wild-type EPEC gradient, in that more of the protein could be found in the lighter fractions (Fig. 4D). These results may indicate that BfpB can influence the membrane localization of BfpU.

Cross-linking and affinity purification of protein complexes

Based on the results of the fractionation and localization experiments which suggest that BfpB influences cellular location of BfpG and may influence the localization of BfpU, we hypothesized that BfpB functions as a central component of an OM subassembly, and probably interacts not only with BfpG and BfpU, but also with periplasmic domains of proteins of the BFP machine. Therefore, potential protein interactions at the OM–periplasm interface were investigated to identify these proteins. We conducted chemical cross-linking with DSP, followed by Western blotting to identify components of BfpB, BfpU and BfpG complexes. Cross-linked proteins were purified from bfpU, bfpG and bfpB mutants complemented with plasmids encoding functional BfpU–His, BfpG–His and BfpB–Strep proteins, respectively. The complexes were purified by affinity chromatography and separated by SDS-PAGE in the presence and absence of the reducing agent β-ME. The gels were either silver-stained (Fig. 5A) or transferred onto PVDF filters and analysed by Western blotting with antibodies against specific Bfp proteins (Fig. 5B).

Silver-stained gels revealed that proteins were present in the eluate fractions, but not in the wash fractions, from all strains except the wild-type EPEC strain E2348/69 (Fig. 5A). Since E2348/69 lacks the affinity tags, this control indicates that the eluted proteins bound specifically to the column with negligible non-specific binding. The elution profile in the β-ME-containing lanes appeared to be similar for BfpG–His and BfpG–His-purified complexes, and different for the BfpB–Strep-purified complex (Fig. 5A). These results suggest that the individual components in BfpU–His and BfpG–His complexes may be similar, while the BfpB–Strep complex may contain an additional subset of BFP proteins.

To identify the cross-linked proteins in the BfpG, BfpU and BfpB complexes, Western blot analyses were conducted with available anti-bundlin, anti-BfpB, anti-BfpG and anti-BfpU antibodies (Fig. 5B). The BfpG–His complex, when probed with anti-BfpB and anti-BfpU antibodies, revealed bands corresponding to BfpB and BfpU in the flow-through and eluate fractions (Fig. 5B, left panel). However, when the BfpG–His complex was probed with anti-bundlin and anti-BfpC antibodies, the bands corresponding to bundlin and BfpC were present in the flow-through but not in the eluate fractions (Fig. 5B, left panel). These results suggest the presence of a complex composed of BfpB, BfpU and BfpG proteins that does not include bundlin or BfpC. Alternatively, there could be separate BfpG–BfpU and BfpG–BfpB complexes, or there could be complexes that include BfpC and bundlin that we could not detect.

In contrast to the results obtained with the purified BfpG–His complex, a band corresponding to bundlin was present in the β-ME-containing flow-through and eluate lanes when the BfpU–His complex was probed with anti-bundlin antibodies (Fig. 5B, middle panel). Additionally, a band with an Mr of ~30 000 was present in the non-reduced eluate lane when probed with anti-bundlin antibodies (Fig. 5B, middle panel asterisk). A similar band of ~30 kDa was also seen when the BfpU–His complex was probed with anti-BfpU antibodies (Fig. 5B, middle panel asterisk; note that a band in the reduced lane migrates slightly slower than the indicated band and likely represents a different protein). This band could possibly be a 1 : 1 complex of bundlin and BfpU, as the relative mobility was similar to that of the sum of those of BfpU and bundlin. When the BfpU–His complex was probed with anti-BfpB and -BfpG antibodies, bands corresponding to BfpB and BfpG were present in the flow-through and eluate fractions (Fig. 5B, middle panel). However, when probed with anti-BfpC antibodies, the corresponding BfpC band was present in the flow-through but not in the eluate fraction (Fig. 5B, middle panel). These results indicate that BfpU exists in close proximity to
bundlin, BfpB and BfpG. Additionally, BfpU may form separate complexes with bundlin and BfpG/BfpB, since bundlin was not detected when BfpG was tagged (above). This latter possibility is compatible with the 30 kDa bands detected in the cross-linked lane of both anti-bundlin and anti-BfpU blots.

The results of the Western blot analyses of BfpB–Strep complex probed with anti-bundlin, anti-BfpC, anti-BfpU and anti-BfpG antibodies are shown in Fig. 5(B), lower panel. The bands corresponding to bundlin, BfpC, BfpU and BfpG were present in the flow-through and eluate fractions of the BfpB–Strep complex, indicating that BfpB exists in close proximity with all these proteins. The presence of BfpC in the BfpB complex is unique to the BfpB cross-linked proteins, and confirms previously published results (Hwang et al., 2003).

Full analysis of all the proteins potentially present in complexes composed of proteins cross-linked to BfpU, BfpG

![Fig. 5. Silver staining and Western blot analyses of affinity-purified, chemically cross-linked complexes. (A) Silver-stained gels of wash (W) and eluate (E) fractions of chemically cross-linked complexes purified by affinity chromatography from (I) wild-type strain E2348/69, (II) complemented bfpG mutant UMD928(pWS16) expressing BfpG–His, (III) complemented bfpU mutant UMD922-(pMSD235) expressing BfpU–His, and (IV) complemented bfpB mutant UMD923-(pWS15) expressing BfpB–Strep, separated in the presence (+) or absence (−) of β-ME. (B) Flow-through (F), wash (W) and eluate (E) samples from chemically cross-linked complexes, affinity-purified by virtue of the tagged proteins indicated at the top of each column, were separated by SDS-PAGE in the presence (+) or absence (−) of β-ME, transferred to PVDF filters, and analysed with the antibodies indicated on the right.](image-url)
and BfpB was limited by the availability of specific antibodies against additional Bfp proteins. Therefore, we were unable to confirm the presence of additional proteins (Hwang et al., 2003).

**Interactions among OM components BfpG, BfpB and BfpU in yeast**

Since chemical cross-linking experiments revealed that complexes purified by virtue of BfpB, BfpG or BfpU each contained the other proteins, we hypothesized that BfpG is not only in close proximity, but interacts directly with BfpB and BfpU. Similarly, BfpU may interact directly with BfpB. To test these hypotheses, a yeast two-hybrid system was used to study the individual interactions of BfpB with BfpG, BfpB with BfpU, and BfpG with BfpU. BfpB, BfpG and BfpU were fused in-frame with both the GAL4 DNA-binding domain and the GAL4 activation domain. The respective plasmid derivatives were co-transformed into *Saccharomyces cerevisiae* strain AH109 and screened for expression of three reporter genes *his3*, *ade2* and *mel1*, which are under the control of a GAL4-inducible promoter, and are expressed if the fusion proteins interact. Yeast expressing interacting fusion protein pairs grew as blue colonies on selective media containing X-α-Gal and lacking tryptophan, leucine, histidine and adenine (−4AA). Our results revealed interactions between murine p53 and SV40 large T-antigen (positive control) and between BfpG and BfpB (Fig. 6). The interactions were confirmed by a quantitative α-galactosidase (α-Gal) assay (Fig. 6). However, there was no evidence of interaction between BfpG and BfpU, since the co-transformants, similar to the negative control, were unable to grow on the selective −4AA plates. When the BfpB and BfpU interaction was tested, there was no growth on the selective −4AA plates but intermediate activity was seen in the α-Gal assay (Fig. 6). The intermediate α-Gal activity, and absence of growth on selective −4AA plates, suggest that the potential interaction between BfpB and BfpU in this system might be of low affinity, or transient. Importantly, all colonies of transformants containing individual constructs were white on medium containing X-α-Gal and lacking tryptophan, or containing X-α-Gal and lacking leucine, verifying that there was no autoinduction of the reporter genes in the absence of the interacting partners (data not shown). Interactions between BfpB and BfpG and many other components of the BFP biogenesis machine, including BfpD, BfpF, the cytoplasmic amino terminus and periplasmic carboxyl terminus of BfpC, and the cytoplasmic amino terminus and two periplasmic loops of BfpE, were also investigated. No evidence of an interaction between BfpB and any of these proteins was detected (data not shown). Thus, the presence of BfpC in the cross-linked complex purified using BfpB–Strep may not have been due to direct interaction between the two proteins.

BfpB belongs to the secretin family of OM proteins (Ramer et al., 1996). Secretins are predicted to be rich in β-pleated sheets that are presumed to fold to form transmembrane β-barrels (Koebnik et al., 2000). Secondary structure analysis

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**Fig. 6.** Yeast two-hybrid analyses. Construct pairs in vectors pGADT7 and pGBK7, respectively, were co-transformed into yeast strain AH109 (only the Bfp protein encoded on vectors pGADT7 and pGBK7, respectively, is indicated). Blue growth on medium containing X-α-Gal and lacking histidine and adenine indicated transcriptional activation of reporters, *his3*, *ade2* and *mel1*. Activation was quantified by measuring α-Gal activity. White bars represent transformants that resulted in growth of blue colonies on selective medium, while those that did not are shown as black bars. Columns denote the mean ± SEM α-Gal activity from three experiments, each performed in triplicate.
of BfpB revealed that β-sheets were present predominantly in the carboxyl-terminal two-thirds of the protein, beginning with residue 172, while few β-sheets were predicted in the amino-terminal third of the mature protein. Thus, we hypothesized that BfpB19–171 is not part of this β-barrel structure, but is present in the periplasmic space and contains possible BfpG and BfpU binding sites. We reasoned that folding of the BfpB structure may have interfered with its translocation to the yeast nucleus, and thus reduced our ability to detect an interaction with BfpU. To determine whether BfpG or BfpU can interact with the amino terminus of BfpB, sequences of bfpB encoding BfpB19–171 and BfpB172–553 were cloned in-frame with both the GAL4 DNA-binding domain and the GAL4 activation domain. These constructs were co-transformed with BfpG and BfpU expression plasmids, and growth was assayed on selective −4AA media containing α-Gal. Growth of co-transformants on selective −4AA plates, as well as the quantitative α-Gal assay, revealed that BfpB19–171 interacted with itself and both BfpG and BfpU, while no interaction between BfpB172–553 and BfpG could be detected (Fig. 6).

**Purified BfpU and the amino-terminal third of BfpB interact in vitro**

We used ITC to characterize the binding of purified and refolded BfpB19–171 to purified BfpU. The ITC profiles resulting from injection of BfpB19–171 into a solution of BfpU at 37 °C are shown in Fig. 7. Each of the spikes in the top panel of Fig. 7 corresponds to a single BfpB injection. The areas under these spikes were determined by integration to yield the associated injection heats. These injection heats were corrected by subtraction of the corresponding dilution heats derived from the injection of identical amounts of BfpB19–171 into buffer alone. The bottom panel of Fig. 7 shows the corrected injection heats for the titration of BfpB into BfpU plotted as a function of the BfpB/BfpU ratio. In this panel, the data points reflect the corrected experimental injection heats, while the line reflects the calculated fit of the data with a model for one set of binding sites. Consistent with the yeast two-hybrid results, the binding parameters extracted from the binding isotherm show that one molecule of BfpB and one of BfpB19–171 interact with high affinity (K_a = 7.414 × 10^6 ± 2.242 × 10^4 M^−1; n = 9.687 ± 3.36 × 10^−3), and indicate that complexes in which BfpB is directly bound to BfpB are biologically plausible (K_a = 135 mM). In addition, the ΔH° of −9.232 ± 4.542 × 10^° cal mol^−1 (3.263 × 10^6 ± 1.900 × 10^4 J mol^−1) shows that the binding of the amino-terminal third of BfpB to BfpU is exothermic.

**DISCUSSION**

Tfp are homopolymeric filamentous structures expressed as surface appendages by many important pathogenic micro-organisms. Tfp function in diverse processes and are assembled by a multi-component molecular machine. Proteins of the Tfp biogenesis machine share similarity to components of protein secretion and DNA uptake systems. EPEC express one such Tfp called BFP, which is assembled and retracted by proteins encoded by the bfp operon. The BFP system is an excellent model for the study of Tfp, as recombinant bacteria transformed with the bfp genes express pili (Stone et al., 1996). Individual components of the BFP machinery have been identified and some of the components have been extensively characterized (Ramboarina et al., 2005; Crowther et al., 2004, 2005; Blank & Donnenberg, 2001). However, structural and functional characterization of the complete BFP biogenesis machine remains to be accomplished.

The inner-membrane (IM) subassembly consisting of BfpC, BfpD, BfpE and BfpF has been characterized in some detail (Crowther et al., 2004). In this study, we attempted to characterize the OM subassembly, and included in our studies the secretin BfpB and two other proteins, BfpG and BfpU, which are present at the OM–periplasm interface. By using
biochemical, biophysical and genetic techniques, we confirmed previous studies that indicate that BfpB and BfpG interact with one another, and that BfpB can be cross-linked to BfpG, BfpU, BfpC and bundlin (Schmidt et al., 2001; Hwang et al., 2003). In addition, we show, to the best of our knowledge for the first time, that BfpB targets BfpG and possibly influences the localization of BfpU within the cell. In the absence of BfpB, BfpG is a periplasmic protein, while targeting of BfpU appears to be altered. By using a yeast two-hybrid system, we provide, as far as we are aware, the first evidence that BfpG and BfpU bind directly to BfpB, and furthermore, that these proteins both bind to the amino terminus of BfpB. Finally, we confirm the BfpU–BfpB interaction by showing that purified BfpU binds with relatively high affinity in vitro to the purified amino terminus of BfpB. Future studies will have to take into account the role of the peptidoglycan in interactions in type IV assembly systems.

BfpG is a small protein encoded by the second ORF of the bfp operon. When the bfp operon was originally described, we noted that the predicted start codon was GTG and we speculated that BfpG might be a lipoprotein, owing to the presence of a critical cysteine after a possible signal peptidase II cleavage site (Stone et al., 1996). However, in this study, we conclusively exclude this possibility by demonstrating that site-directed mutagenesis of this cysteine residue has no effect on BfpG size or function. These data confirm those of Schmidt et al., who were unable to detect incorporation of labelled palmitic acid into BfpG (Schmidt et al., 2001). Furthermore, we show that purified BfpG exists in two forms after cleavage at either of two typical signal peptidase I sites, as determined definitively by amino-terminal amino acid sequencing.

In cross-linking assays, we found that BfpG was present in BfpB-purified complexes and, reciprocally, BfpB was present in BfpG-purified complexes, confirming prior observations (Schmidt et al., 2001; Hwang et al., 2003). Additionally, BfpG interacted with full-length BfpB in the yeast two-hybrid assay, which provides for the first time, as far as we know, evidence that BfpB and BfpG interact directly with one another, rather than merely being part of the same complex. Further refinement suggests that there is a direct interaction between BfpB and the amino-terminal one-third domain of BfpB. Schmidt et al. (2001) reported that BfpG is required for the formation and/or stability of BfpB multimers because, in a bfpG mutant, BfpB is present predominantly as a monomer. However, we found that formation of BfpB multimers did not strictly require BfpG, since BfpB ring structures were also observed for purified E. coli K-12 BfpB in the absence of BfpG (data not shown). However, it remains possible that BfpG is required for BfpB multimerization in EPEC, but not in E. coli K-12, owing to the presence or absence of additional proteins that affect this process.

We investigated whether the absence of BfpG alters the membrane localization of BfpB. Previous studies have indicated that small lipoproteins, usually encoded adjacent to their cognate secretins, pilot secretins to the OM (Crago & Koronakis, 1998; Drake et al., 1997; Hardie et al., 1996; Shevchik et al., 1997). Although BfpG is not a lipoprotein, it is of similar size to these pilotins, and is encoded immediately upstream of bfpB. Our membrane purification and sucrose density gradient experiments reveal that BfpB is targeted to the OM (and CM when overexpressed in an E. coli K-12 background) independent of BfpG, thus confirming earlier results that BfpG is not a pilotin (Schmidt et al., 2001). We also investigated whether the converse is true, i.e. whether the absence of BfpB alters the targeting of BfpG to the OM. Our results indicate that BfpG is a peripheral OM protein that is targeted to that location by BfpB. In the wild-type EPEC strain, BfpG was present in the insoluble membrane fraction, which corresponds to the OM as determined by sucrose density gradient experiments, while, in the bfpB mutant, BfpG was present exclusively in the soluble fraction corresponding to the periplasm/cytoplasm. In the periplasmic separation assays, BfpG was detected primarily in the periplasmic fraction of the bfpB mutant. This result is consistent with the cleavage of BfpG by signal peptidase I and with the overall hydrophilic nature of the mature protein. Additionally, when the bfpB mutant was complemented with functional BfpB protein, BfpG was restored to fractions corresponding to the OM in density gradient fractionation. The above results conclusively prove that targeting of BfpG to the OM is dependent on BfpB. Interestingly, other investigators have failed to detect BfpG in the absence of BfpB, and have thus been unable to determine the effect of BfpB on BfpG localization (Ramer et al., 2002). We speculate that our ability to detect BfpG, and in turn determine the effect of BfpB on BfpG localization, may be due to the nature of the bfpB mutant used for these studies. The mutant that we used is the result of the disruption of the locus with a non-polar gene cassette (Anantha et al., 2000). We could detect the amino terminus of BfpB in this mutant using an antiserum raised against an amino-terminal peptide (data not shown). It is therefore likely that an intact BfpB protein is required for BfpG recruitment to the OM, while fragments of BfpB are sufficient to maintain BfpG stability.

The relationship between BfpB and BfpG differs in many respects from other known secretin systems, such as the PulD–PulS of Klebsiella, OutS of Erwinia, InvG of N. enterica serotype Typhimurium, as well as PilQ of N. gonorrhoeae and P. aeruginosa, as has been discussed in detail by Schmidt et al. (2001). First, the secretin BfpB is a lipoprotein similar to XpsD from Xanthomonas campestris of the pIV–PulD superfamily of OM secretin secretins (Ramer et al., 1996; Genin & Boucher, 1994; Yen et al., 2002), while the partner BfpG is not a lipoprotein. However, in other members of the pIV–PulD superfamily, the converse is true. Second, the small lipoprotein plays a role in the targeting/stability of secretins. In the BfpB–BfpG system, BfpB is not required for the targeting/stability of BfpB. However, as far as we are aware, our study demonstrates for the first time that the BfpB secretin is required to recruit the small interacting
protein BfpG to the OM. Since vancomycin sensitivity assays suggest that BfpB forms an incompletely gated channel, a possible function of BfpG is to function as the gate of the BfpB channel.

Hwang et al. (2003) demonstrated that BfpB cross-links with at least nine BFP proteins, including BfpC. BfpC, along with BfpD, BfpE and BfpF, constitutes the CM subassembly (Crowther et al., 2004). The presence of BfpC in BfpB cross-linked complexes led Hwang et al. (2003) to postulate that the BFP machine spans the periplasmic space. In our cross-linking assays, we detected BfpC only in BfpB-purified complexes but not in BfpG- or BfpU-purified complexes, suggesting that these complexes are not identical. These results also support the hypothesis that the BFP machine spans the periplasmic space via a BfpB–BfpC interaction. However, when tested in the yeast two-hybrid assay, we did not observe an interaction between BfpB and BfpC (data not shown). Although the two-hybrid results do not prove that there is no interaction between BfpB and BfpC, we obtained similar results with BfpG and BfpU (discussed below). Thus, currently, it is not clear how the OM and CM subassemblies of the BFP machine are linked.

The distribution of BfpU in the cell is more complex than that of any other BFP protein. BfpU is present in the periplasm and cytoplasm (Schreiber et al., 2002), as well as in the CM (Ramer et al., 2002). Our membrane fractionation experiments confirm earlier observations that BfpU is present in the soluble, as well as the membrane fractions. The periplasmic fractionation experiments demonstrate that part of the BfpU pool is periplasmic. In sucrose density gradient fractions, we found that BfpU was present in the bottom of the sucrose gradient (which contains soluble and aggregated proteins), as well as in the fractions corresponding to the OM. Additionally, the absence of BfpB appears to alter the distribution profile of BfpU compared to wild-type EPEC, while, in the complemented bfpB mutant, the distribution of BfpU appears to be similar to that of wild-type EPEC, indicating that BfpB may also influence the targeting of BfpU within the cell.

Cross-linking assays revealed that BfpU-purified complexes contain BfpB and BfpG. Similarly, BfpU was detected in both BfpB- and BfpG-purified complexes, suggesting that BfpB, BfpU and BfpG are in close proximity to each other. When the interactions were tested in the yeast two-hybrid assay, the strain containing full-length BfpB and BfpU did not grow on selective plates, but exhibited intermediate a-Gal activity, which could have been due to weak or transient interaction between these proteins. However, the amino-terminal one-third domain of BfpB interacted strongly with BfpU. This result suggests that the carboxyl-terminal two-thirds of BfpB inhibits the interaction with BfpU in yeast, yielding an intermediate result. Furthermore, using ITC, we confirmed that BfpU binds directly to the amino-terminal third of BfpB. This interaction was equimolar and strong (dissociation constant $1.35 \times 10^{-7} \text{ M}$). Although these in vitro studies do not simulate conditions present in the native OM environment, the relatively low dissociation constant of this interaction is indicative of strong binding, suggesting that the BfpB–BfpU complex is likely to be present at concentrations found in the bacterial cell. These results further support the notion that BfpU is a component of the OM subassembly. In contrast, BfpU and BfpG did not interact in the two-hybrid assay, and we were also unable to detect any binding between purified BfpU and BfpG by ITC (data not shown), suggesting that, even though both BfpG and BfpU are in close proximity and can be cross-linked, the proteins may not interact with each other directly. Thus, the results of the targeting experiments, as well as those of the interaction studies, indicate that BfpU is a component of the OM subassembly, but also suggest that BfpU can be found in all other compartments as well, especially in the absence of BfpB.

Another novel observation of the cross-linking assay is that bundlin was detected in BfpU-purified complexes. The results of the cross-linking assay do not prove a direct interaction between BfpU and bundlin, since additional proteins might serve as intermediaries. Nevertheless, the presence of both bundlin and BfpU in the CM, as well in the OM, and the cross-linking between bundlin and BfpU suggest that BfpU could play a role in the delivery of bundlin to the OM. The presence of BfpU in multiple compartments of the cell, and the interaction of BfpU with components of the OM assembly, as well as components of the CM subassembly (L. J. Crowther and others, unpublished observations), suggest that BfpU plays a dynamic role in the BFP biogenesis process.

In this study, we investigated the interactions among three BFP proteins and with various components of the BFP machine, in an attempt to gain insight into the roles that these proteins play in the BFP biogenesis process. Our results indicate that BfpB, BfpG and BfpU are a part of the OM subassembly of the BFP biogenesis machine. BfpB appears to be the central component of the OM subassembly, forming a ring with a central channel through which the pilus fibres likely pass. BfpB also targets BfpG and BfpU to the OM through direct interactions between these proteins and its amino terminus. The complex distribution pattern, as well as the multiple interactions of BfpU, indicate that it plays a dynamic role in the pilus biogenesis process. Ongoing investigations in our laboratory are aimed at defining this role.

ACKNOWLEDGEMENTS

We thank Steven Munger for assistance with the isothermal titration calorimeter. This work was supported by a Public Health Service grant (R01 AI-37606).

REFERENCES


its relationship to bacterial type IV pili and archaeal flagella. Microbiology 149, 3051–3072.