A Novel Approach to Mapping Protein Interactions During Pilus Biogenesis by Using *in vivo* Photocrosslinking

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ABSTRACT

The purpose of this study was to map the protein interactions involved in uropathogenic Escherichia coli P pilus secretion. P pili are external structures that facilitate adhesion to kidney epithelial cells, playing a major role in bacterial pathogenesis. Constructed through the chaperone/usher pathway, they are composed of monomeric proteins that are assembled and secreted at the cell surface by an outer membrane usher. The N-terminal domain of the usher contains a disulfide loop region that is required for unidentified steps of pilus assembly following the binding of chaperone-subunit complexes. To map interactions of the disulfide loop region, mutant amber suppressor tRNA was used to incorporate a synthetic photocrosslinkable amino acid, p-benzoylphenylalanine (pBpF), at sites distributed throughout the loop region. Interactions were captured upon exposure to UV light. Results show that the photocrosslinking method is functional for use in the study of P pilus biogenesis, that pBpF can be incorporated without structural deformation, and that residues Y91 and D94 are involved in *in vivo* interactions. Understanding the mechanisms of the chaperone/usher pathway can lead not only to a further understanding of bacterial pathogenesis, but also to the development of novel methods for fighting bacterial infection.

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INTRODUCTION

This project involved the novel use of photocrosslinking in an attempt to map *in vivo* protein interactions during pilus biogenesis. This method has not previously been applied to mapping protein interactions in pilus assembly; prior studies were limited to *in vitro* analysis or alanine substitution mutations, which can only identify important residues and not with which proteins the residues are interacting. The motivation for using this technique was to increase our understanding of the molecular mechanisms of pilus biogenesis. In doing so, the process of bacterial attachment to host cells could be elucidated. With the current tendency towards antibiotic overuse and the development of bacterial resistance to antibiotics (Olson *et al.*, 2009), it has become imperative to understand the mechanisms of bacterial pathogenesis. The ultimate goal of this research direction is to develop novel therapeutic methods involving pilus disruption that can be used to treat bacterial infections. Statistics show that one half of American women will contract at least one urinary tract infection during their lifetime, leading to yearly health care costs of $2 billion (Foxman & Brown, 2003).

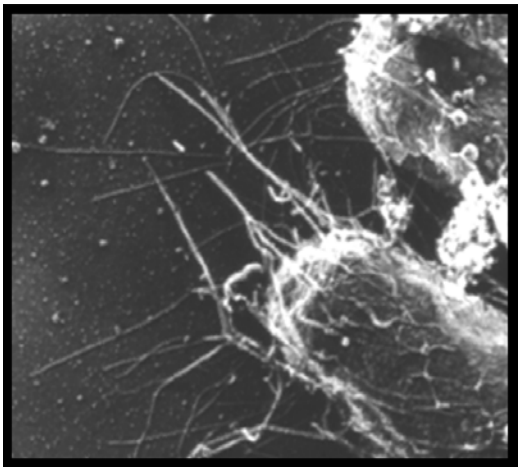

Fig. 1. Electron micrograph of *Escherichia coli* expressing P pili (Thanassi & Hultgren, 2000).

A crucial step in infection is host cell recognition and attachment, a function that is carried out by adhesive organelles known as pili (Fig. 1). Uropathogenic *Escherichia coli* (*E. coli*) and various other pathogens use the chaperone/usher (CU) pathway to assemble pili known as P pili, encoded by the *pap* gene cluster (Thanassi *et al.*, 1998a). P pili are responsible for bacterial colonization of the kidney; by binding to receptors in the kidney, they tether the bacteria to the cells and allow *E. coli* to cause the disease pyelonephritis (Roberts *et al.*, 1994). The P pilus consists of a tip fibrillum connected to a helical rod (boxed, Fig. 2) (Kuehn *et al.*, 1992; Bullitt & Makowski, 1995).

Pilus assembly requires two components that are not part of its final structure: the periplasmic chaperone (PapD) and the outer membrane (OM) usher (PapC) (Thanassi *et al.*, 1998a). All proteins, for both structure and assembly, cross the inner membrane (IM) into the

periplasm through the Sec secretory pathway (Fig. 2) (Pugsley, 1993). Pilus structural subunits then interact with the chaperone (PapD) to form a stable complex (Vetsch *et al.*, 2004). The structure of the chaperone allows the pilus subunit to fold properly by donating structural components, in a process known as donor-strand complementation (Sauer *et al.*, 1999). A similar process occurs at the OM usher, where each consecutive subunit performs donor-strand exchange to replace the chaperone, in a process that results in a lower energy state, thus providing the driving force for pilus fiber biogenesis (Sauer *et al.*, 2002; Zavialov *et al.*, 2005).

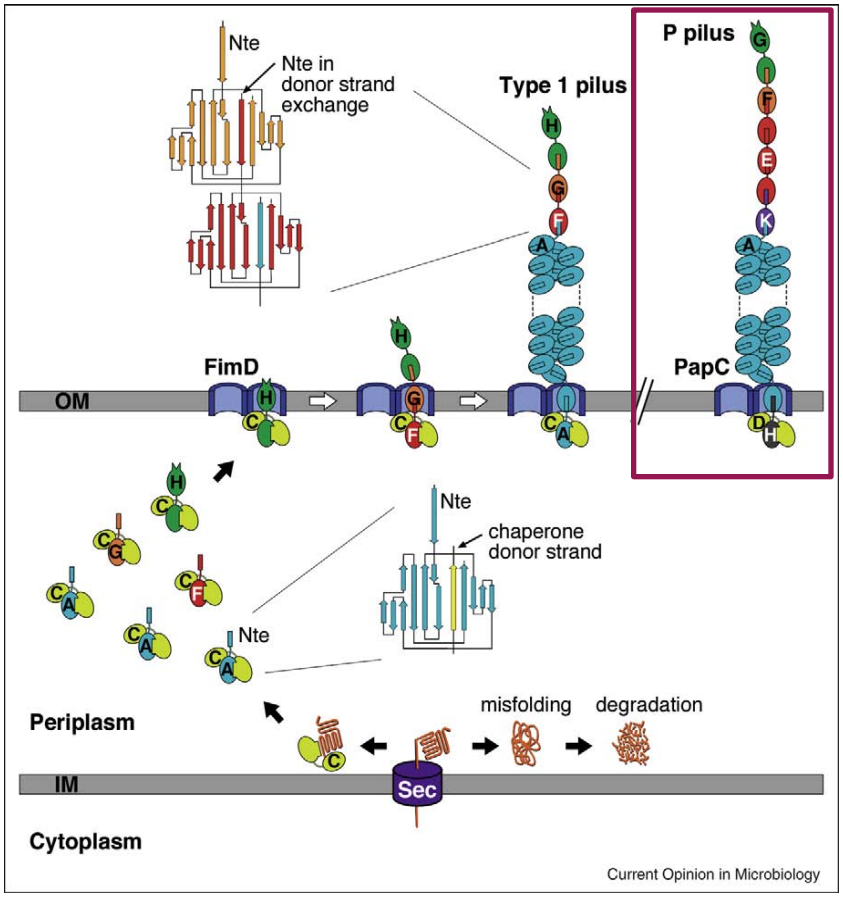

Fig. 2. Model for pilus biogenesis by the CU pathway.
Donor-strand complementation and donor-strand exchange are shown for assembly and secretion of the Type 1 pilus, which is analagous in structure and assembly to the P pilus. Proteins are indicated by letter (A for PapA, G for PapG, etc.). The assembled P pilus is shown at right, boxed (Li & Thanassi, 2009).

Although much is known about chaperone-subunit and subunit-subunit interactions, information still remains to be uncovered about the interactions of the OM usher, especially in regard to its catalytic ability. The OM usher functions as an asymmetric twin-pore dimer, with pilus subunits being assembled through only one of the pores (Fig. 3A) (Li *et al.*, 2004; Remaut

et al., 2008). Chaperone-subunit complexes are recruited by the N-terminal domain of the usher in order of final assembly position (Ng *et al.*, 2004; Dodson *et al.*, 1993). After N-terminal targeting, the C-terminal domain stably binds the chaperone-subunit complex to the usher (Shu Kin So & Thanassi, 2006). A plug domain prevents unintended secretion of pilus subunit proteins (Remaut *et al.*, 2008).

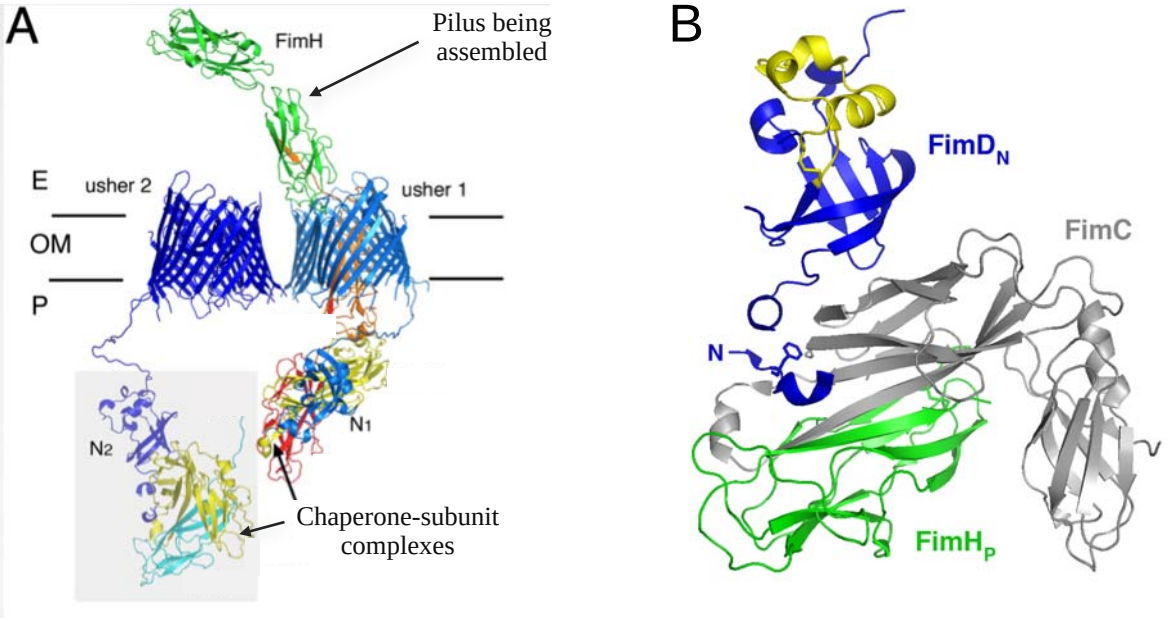

Fig. 3. (A) 3D Cryo-EM Reconstruction of the FimD2:C:F:G:H Type 1 Pilus Tip Assembly Intermediate (Remaut *et al.*, 2008). (B) Ribbon diagram of FimD N terminus interacting with chaperone-subunit complex. Disulfide loop region highlighted in yellow (based on data from Nishiyama *et al.*, 2005).

While the N-terminal domain has been shown to be vital for the initial recruitment of chaperone-subunit complexes, it was also found to be needed at a currently unidentified later stage of pilus biogenesis (Ng *et al.*, 2004). Residues 2-11 of the N terminus are required to bind chaperone-subunit complexes, residue F3 being of particular importance (Ng *et al.*, 2004). These residues are necessary because they form the interface for interaction with chaperone-subunit complexes (Nishiyama *et al.*, 2005). The N terminus also contains a disulfide loop region bounded by cysteine residues at positions 70 and 97 (Fig. 3B) (Henderson *et al.*, 2004; Nishiyama *et al.*, 2005). This disulfide loop region is required at a later stage of pilus assembly, but its precise function is not yet known; a previous crystal structure of an analogous pilus assembly system could not identify the region interacting with the bound chaperone-subunit complex (Fig. 3B) (Ng *et al.*, 2004, Nishiyama *et al.*, 2005, Henderson *et al.*, 2011).

This study used *in vivo* site-directed photocrosslinking to solve the *in vivo* protein interactions of the disulfide loop region. The method was originally developed by the Schultz laboratory at The Scripps Research Institute (Young *et al.*, 2010); my study is the first to utilize this technique in understanding biogenesis by the chaperone/usher secretion pathway. By expressing amber mutants of PapC with mutant amber suppressor tRNA and tRNA synthetase, a synthetic photocrosslinkable amino acid, *p*-benzoylphenylalanine (*p*BpF), was incorporated at chosen sites in the OM usher (Young *et al.*, 2010). When the protein expressing *p*BpF was irradiated with UV light, the carbonyl oxygen of the benzophenone group of *p*BpF reacted with nearby carbon-hydrogen bonds (Chin *et al.*, 2002). Results thus far indicate that, with adaptation, the described method can also be applied to the P pilus biogenesis pathway. By using photocrosslinking, the *in vivo* protein interactions of the disulfide loop region during pilus biogenesis can be mapped.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions

The strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown overnight at 37˚C with vigorous shaking in 5 ml Luria-Bertani (LB) growth medium, with 100 µg/ml ampicillin, 50 µg/ml kanamycin, and/or 25 µg/ml chloramphenicol where appropriate. Colonies were grown on 25 µl LB agar plates with appropriate antibiotics. Cultures used to examine protein expression were diluted 1:40 in fresh LB with antibiotics and allowed to grow to an optical density of 0.6 at 600 nm (OD_{600}) before inducing. When indicated, cells were grown with 200 µM *p*BpF (Bachem) upon 1:40 dilution of overnight cultures. Plasmids with arabinose-inducible promoters were induced with 0.1% L-arabinose and those with isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoters were induced with 50 µM IPTG, both for 1 hour.

Amber mutants were derived from pDG2, which contains a wild-type (WT) PapC sequence followed by a C-terminal thrombin cleavage site and a hexahistidine tag (His-tag), promoted by an arabinose-inducible promoter (P_{ara}) and ampicillin resistance (Amp^r). Amber mutations (mutation to the amber stop codon TAG) were inserted using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) at positions evenly distributed throughout the disulfide loop region

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[b]	Reference
Strains[a]		
DH5α	*hsdR recA endA*	Grant *et al.* (1990)
SF100	Δ*ompT*	Baneyx and Georgiou (1990)
Plasmids		
pMON6235Δcat	vector, P_{ara}, Amp^r	Jones *et al.* (1997)
pPAP58	PapDJKEFG in pMMB91	Hultgren *et al.* (1989)
pMJ3	PapC with C-terminal hexahistidine (His) tag in pMON6235Δcat	Thanassi *et al.* (1998b)
pDG2	pMJ3 with thrombin cleavage site before His-tag	Li *et al.* (2004)
pEVOL-*p*BpF	Amber suppressor tRNA and aminoacyl-tRNA synthetase derived from *Methanocaldococcus jannaschii*	Young *et al.* (2010)
pEPS73AMB	Amber mutation at residue S73 of PapC in pDG2	This study
pEPV76AMB	Amber mutation at residue V76 of PapC in pDG2	This study
pEPM79AMB	Amber mutation at residue M79 of PapC in pDG2	This study
pEPT82AMB	Amber mutation at residue T82 of PapC in pDG2	This study
pEPS85AMB	Amber mutation at residue S85 of PapC in pDG2	This study
pEPK88AMB	Amber mutation at residue K88 of PapC in pDG2	This study
pEPY91AMB	Amber mutation at residue Y91 of PapC in pDG2	This study
pEPD94AMB	Amber mutation at residue D94 of PapC in pDG2	This study
pEPF3AMB	Amber mutation at residue F3 of PapC in pDG2	This study
pEPN4AMB	Amber mutation at residue N4 of PapC in pDG2	This study

(a) All strains are *E. coli* K-12.
(b) Amp^r, ampicillin resistance; P_{ara}, arabinose-inducible promoter; P_{trc}, IPTG-inducible promoter.

of the N-terminal domain, with DH5α as the host strain. The primers used for mutagenesis are listed in Table 2. All mutants were completely sequenced to ensure the presence of the desired mutation and the absence of unintended mutations. Cells were plated on ampicillin LB agar plates to ensure selection of transformed cells. Selected colonies were then restreaked and their plasmids purified using a Promega Wizard® Plus Miniprep kit (Promega). Presence of the amber mutation was then checked by having the relevant area sequenced with a primer upstream of the mutated region.

Table 2. Primers used in this study

Primer	Sequence[a,b] (5'-3')
S73AMB	CCG CAG GCC TGT CTG ACA TAG GAT ATG GTC AGA CTG
V76AMB	CCG CAG GCC TGT CTG ACA TCA GAT ATG TAG AGA CTG ATG GGG
M79AMB	CTG ACA TCA GAT ATG GTC AGA CTG TAG GGG TTA ACA GCA G
T82AMB	CA GAT ATG GTC AGA CTG ATG GGG TTA TAG GCA GAA TCT CTG G
S85AMB	G GGG TTA ACA GCA GAA TAG CTG GAT AAA GTT GTT TAC TGG CAT G
K88AMB	G GGG TTA ACA GCA GAA TCT CTG GAT TAG GTT GTT TAC TGG CAT G
Y91AMB	GCA GAA TCT CTG GAT AAA GTT GTT TAG TGG CAT GAT GGT CAG
D94AMB	CT CTG GAT AAA GTT GTT TAC TGG CAT TAG GGT CAG TGT GCG G
F3AMB	GCC AGT GCC GTT GAG TAG AAT ACA GAT GTA CTT GAC GC
N4AMB	GCC AGT GCC GTT GAG TTT TAG ACA GAT GTA CTT GAC GCA GCG G

(a) Reverse primer is the reverse complement of the forward primer, as per QuikChange kit protocol.
(b) Amber mutation underlined.
All primers are from this study.

Outer Membrane Isolation and Analysis of Usher Expression and Folding

The outer membrane was isolated through sonication and sarkosyl extraction as described (Ng *et al.*, 2004) using *E. coli* host strain SF100, which lacks the OmpT OM protease (Table 1). Cells contained PapC mutant or control plasmids and/or suppressor tRNA encoding plasmid as indicated. Expression levels of the usher in the OM were determined by immunoblotting with anti-His-tag (Covance) or anti-PapC antibodies. Immunoblots were developed with alkaline phosphatase-conjugated secondary antibodies and BCIP (5-bromo-4-chloro-3-indolylphosphate)-NBT (nitroblue tetrazolium) substrate (KPL). Proper folding of the ushers in the OM was checked by resistance to denaturation by SDS, which provides an indication of the correct folding and stability of the ß-barrel domain (Sugawara *et al.*, 1996). This resistance was determined by heat-modifiable mobility on SDS-PAGE, performed as previously described (Ng *et al.*, 2004; Shu Kin So and Thanassi, 2006).

In Vivo Photocrosslinking

The reported method (Okuda & Tokuda, 2009) was slightly modified. Strain SF100 was used as host strain. Plasmid pPAP58, encoding the pilus tip subunits and chaperone (PapDJKEFG), and pEVOL-*p*BpF, encoding amber suppressor tRNA and aminoacyl-tRNA synthetase, were transformed into the cells along with pMON6235Δcat vector, pDG2 WT PapC, or mutant plasmid. Cells were grown as described above. Aliquots (200 µL) of the cultures were transferred to microtiter plates, followed by irradiation with UV light at 365 nm for 5 min and 13 cm away from the light source or for 7 min and 3.3 cm away by using B-100AP (UV Products) at room temperature. The cells were harvested by centrifugation at 9,000 x g for 2 to 3 min, resuspended in SDS-PAGE buffer, boiled at 95˚C for 10 minutes, and then analyzed by SDS-PAGE and immunoblotting with antibodies against the specified proteins.

RESULTS

Construction of Amber Mutants

Site-directed mutagenesis was used to introduce the amber stop codon (TAG) at positions evenly distributed throughout the loop region of the N-terminal domain. Amber mutations were also introduced at residues 3 and 4, as these residues have been confirmed to bind PapDG chaperone/adhesin complexes (Ng *et al.*, 2004; Henderson *et al.*, 2011). Proper construction of

the mutants was confirmed by comparing sequences of sections of DNA to the intended plasmid using Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information (NCBI)).

The pEVOL-*p*BpF plasmid containing amber suppressor tRNA and aminoacyl-tRNA synthetase was transformed into *E. coli* SF100 and confirmed by antibiotic resistance. Since pEVOL-*p*BpF contains the same origin of replication (p15A) as the Δ *papC pap* plasmid normally coexpressed with usher-containing plasmids, plasmid pPAP58, containing only pilus tip subunits and chaperone (PapDJKEFG), was used in its place to assess the ability of the usher to assemble pili. Additionally, while AAEC185, a strain lacking the *fim* gene cluster that codes for type 1 pili, is normally used in complementation assays (P pili have been previously shown (Ng *et al.*, 2004) to be able to be constructed through the type 1 pilus pathway), AAEC185 contained amber suppressor tRNA activity, making it unusable for this study (data not shown). Experiments were performed (data not shown) to ensure that type 1 pili were not being assembled. Plasmid pPAP58 was then introduced into SF100 cells containing pEVOL-*p*BpF, and confirmed by antibiotic resistance.

Incorporation of Unnatural Amino Acid

Amber mutants and controls were then expressed in SF100 cells under two sets of conditions. The first set was conducted with presence or absence of pEVOL-*p*BpF, presence of *p*BpF, and absence of pilus tips (Fig. 4A). The second set was conducted with presence or absence of *p*BpF, presence of pEVOL-*p*BpF, and presence of pilus tips (Fig. 4B). Incorporation of the unnatural amino acid into the PapC proteins was confirmed through harvesting bacteria grown as indicated, denaturing by SDS, running gel electrophoresis, and immunoblotting with anti-His antibody. Of note is the presence of a low level of usher protein in cultures grown without *p*BpF (Fig. 4B); this will later be discussed in further detail.

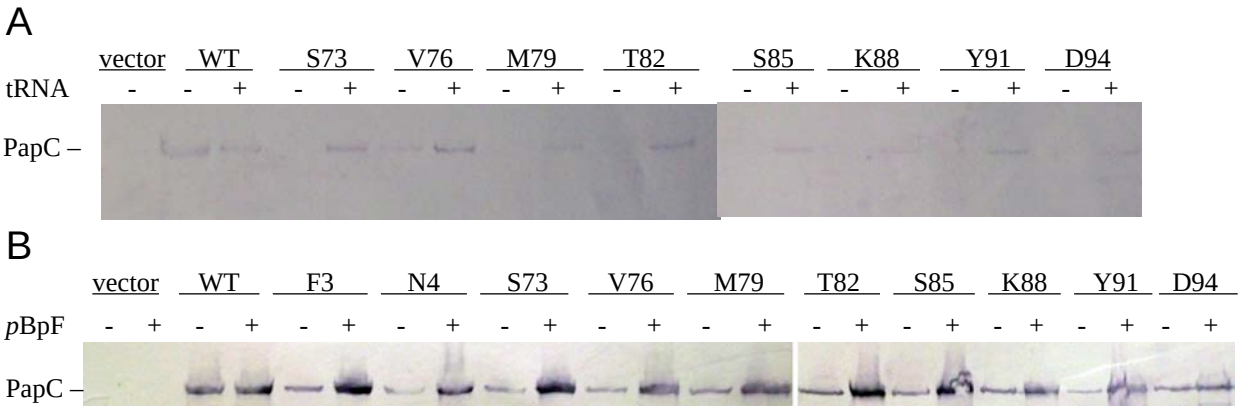

Fig. 4. (A) Expression of amber mutants of PapC in the presence or absence of amber suppressor tRNA, analyzed by SDS-PAGE and immunoblotting with anti-His antibody. (B) Expression of amber mutants of PapC in the presence or absence of unnatural amino acid *p*BpF, analyzed by SDS-PAGE and immunoblotting with anti-His antibody.

Usher Expression, Folding, and Oligomerization

Before using the PapC amber mutants in photocrosslinking experiments, the proper expression and folding of the ushers needed to be confirmed. Cells harboring pEVOL-*p*BpF and amber mutant usher plasmids were grown in the presence of *p*BpF and the outer membrane was prepared as previously described. Blotting with anti-His antibody showed that, compared to wild-type usher, all mutants expressed and folded properly (Fig. 5).

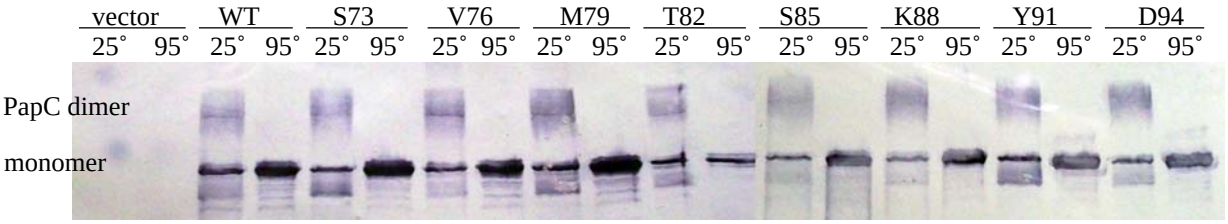

Fig. 5. Outer membrane isolation and temperature shift assay. Amber mutants of PapC expressed in the presence of amber suppressor tRNA and *p*BpF, incubated in SDS at 25˚C or 95˚C as indicated, and analyzed by SDS-PAGE and immunoblotting with anti-His antibody. If properly folded, dimer is stable at 25˚C and monomer is stable at 95˚C.

Usher Interactions in Presence of Pilus Tip Subunits

SF100 cells harboring pEVOL-*p*BpF, pPAP58, and amber mutant usher plasmids were grown in the presence of *p*BpF, irradiated with UV light, harvested, denatured with SDS, and immunoblotted as described. Initially, SDS-PAGE gels were immunoblotted with anti-His antibody that binds to a C-terminal hexahistidine tag present on the parent usher from which the mutants were derived (Fig. 6A). Bands migrating to 88 kDa represented uncrosslinked PapC. Higher bands indicated crosslinking with tip subunits, clearly visible for usher mutants containing amber mutations at residues 3, 4, and 94. In some trials, residue 91 showed a band

running slightly below WT PapC, possibly indicating internal crosslinking (Fig. 6B). The band was most visible when exposed to UV light for 7 minutes at a distance of 3.25 cm.

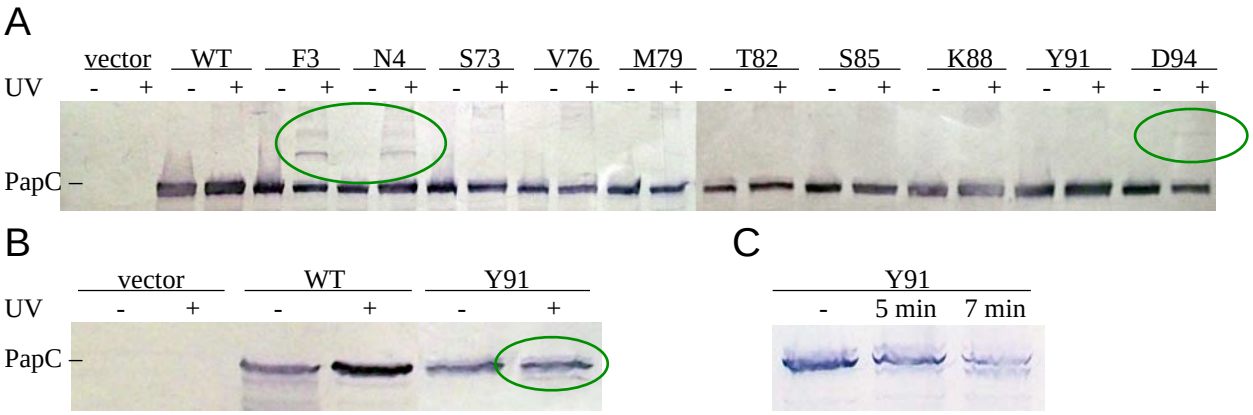

Fig. 6. (A) Expression of amber mutants of PapC in the presence or absence of UV light, analyzed by SDS-PAGE and immunoblotting with anti-His antibody. (B) Expression of residue 91 mutant of PapC in the presence or absence of UV light, analyzed by SDS-PAGE and immunoblotting with anti-His antibody. (C) Expression of residue 91 mutant of PapC under varying levels of UV light, analyzed by SDS-PAGE and immunoblotting with anti-His antibody.

Samples were then analyzed by SDS-PAGE and immunoblotting with anti-P pilus tips antibody, to determine if residues of the cysteine loop region bind to pilus tip subunits (data not shown). However, since the anti-P tips antibody is not as specific as anti-His, there were no distinguishable results; the data from this experiment were inconclusive.

Since anti-tips did not produce clear results, it was hypothesized that this could be due to the antibody binding only to the pilus subunits, and not to the chaperone-subunit complexes bound to the usher. To test this, samples were immunoblotted with anti-PapDG antibody that binds to the chaperone PapD and the terminating tip adhesin PapG (Fig. 7). The results showed a lightness in many of the bands, due to multiple uses of the sample resulting in less protein than usual being run on the gel. While higher-running bands appeared in some samples, further investigation is required for conclusive results.

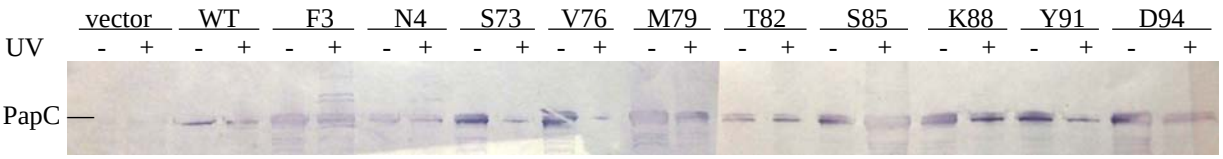

Fig. 7. Expression of amber mutants of PapC in the presence or absence of UV light, analyzed by SDS-PAGE and immunoblotting with anti-PapDG antibody.

DISCUSSION AND CONCLUSIONS

The purpose of this study was to observe the interactions of the disulfide loop region *in vivo* by scanning the region with *p*BpF substitution mutations and photocrosslinking mutated residues, a novel approach to mapping protein-protein interactions of the outer membrane usher. PapC mutant plasmids were successfully constructed, and shown to be capable of expressing *p*BpF with no structural deformations. The photocrosslinking technique was shown to be functional for use in the study of P pilus biogenesis. Based on observations indicating photocrosslinked products at certain residues, the disfulfide loop region likely serves a structural purpose in catalyzing pilus biogenesis.

When PapC was expressed in the presence of *p*BpF and absence of pEVOL-*p*BpF, no PapC protein was made (Fig. 4A), but when expressed in the absence of *p*BpF and presence of pEVOL-*p*BpF (Fig. 4B), PapC protein was produced, albeit at a comparatively low level. Currently, there are two hypotheses for this result. First, at the time the test was performed in the absence of pEVOL-*p*BpF, the anti-His antibody was used at a 1,000-fold lower concentration than later experiments, due to use of antiquated laboratory protocols. Thus, it could be that PapC was being expressed, but was not visible at that level. Alternatively, a different amino acid could have been inserted at that position, indicating that pEVOL-*p*BpF can bind other amino acids, but with less affinity. To determine the cause of this discrepancy, cells will once again be grown in the presence of pEVOL-*p*BpF and absence of *p*BpF and immunoblotted with the proper concentration of anti-His.

While the disulfide loop mutants can be investigated further, evidence indicates that the photocrosslinking method can be used to analyze the P pilus system. Residue F3 of the N-terminal domain has been shown to directly bind the chaperone (Ng *et al.*, 2004; Ng *et al.*, 2006; Nishiyama *et al.*, 2005), providing a positive control that should consistently crosslink with the chaperone PapD. Amber mutants at residues F3 and N4 were able to incorporate *p*BpF (Fig. 4B). When exposed to UV light and analyzed by SDS-PAGE and immunoblotting with anti-His (Fig. 6A), the mutants displayed a ladder of higher bands, indicating that they were bound to various chaperone-subunit complexes. Thus, results obtained by using this method are valid.

Of note in the crosslinked product results is the PapC amber mutant at residue Y91. While previous crosslinking tests had shown the UV-exposed Y91 amber mutant running slightly below unexposed usher (Fig. 6B), one iteration of the crosslinking protocol showed no faster migration (Fig. 6A). To examine why this occurred, the Y91 mutant plasmid was retransformed into the expression strain in the unlikely case of contamination, run on a gel with wider lanes, and exposed to UV light for varying lengths of time and distances from the light source. As indicated in Fig. 6C, a longer exposure time produced a stronger crosslinked band. Thus, the apparent absence of crosslinked product in Fig. 6B was most likely due to low levels of visibility on the immunoblotted gel.

Another residue of note is D94. This residue is conserved in the analogous type 1 usher FimD. In multiple crosslinking experiments conducted in both host strains AAEC185 and SF100, a band running at ~120 kDa appeared in only those samples exposed to UV light (Fig. 6A). This band was not observed when blotted with anti-tips or anti-DG; however, the band was faint when immunoblotted with anti-His, therefore the band is either not present or not strong enough to appear. Initially, it will be prepared again and a stronger sample will be immunoblotted with anti-DG. This demonstrates the need for future avenues of study: optimizing the photocrosslinking protocol; mass spectrometry; and coexpression with individual subunits.

While positive controls F3 and N4 display crosslinked proteins when immunoblotted with anti-His (Fig. 6A), the bands are relatively faint as compared to uncrosslinked protein. Although not all ushers expressed are in the process of interaction at the time of crosslinking, it may be possible to increase the expression of ushers containing *p*BpF or the amount of usher that is crosslinked. Possible conditions to vary include arabinose inducer concentration, length of time exposed to UV light, and *p*BpF concentration.

Since the usher used has a C-terminal hexahistidine tag, it can be purified by using a nickel affinity column. If the volume used for crosslinking is scaled up, protein can be then purified in this manner and subjected to mass spectrometry to ascertain the precise molecular weight of crosslinked products. This can be used to determine to which proteins the usher has crosslinked.

Thus far, the usher has only been coexpressed with tips subunits in crosslinking experiments. To more precisely identify interactions of the disulfide loop region during pilus

biogenesis, mutants can be coexpressed with individual subunits or combinations thereof. Additionally, crosslinking experiments can be conducted in the absence of any subunits to test for crosslinking within the usher or dimerization.

To gain a complete understanding of the effect of the inclusion of *p*BpF in the usher, pilus assembly and function can be assessed. Previous studies have indicated that, while alanine substitution for either of the bounding cysteines or deletion of the entire loop region produced a phenotype negative for pilus assembly (Ng *et al.*, 2004), alanine substitution mutations of individual residues within the loop region produced no distinguishable phenotype from wild-type usher (T. W. Ng, I. Talukder, and D. G. Thanassi; unpublished data). Thus, the amber usher mutants should be able to assemble pili, though this has yet to be tested. Using WT usher, an immunofluorescence assay previously used in *Yersinia pestis* (Runco *et al.*, 2008) will be adapted for use with P pili.

Results thus far indicate that the disulfide loop domain serves a structural purpose. Since the photocrosslinking technique was shown to be effective in the chaperone/usher system by comparison to the known binding sites F3 and N4, negative results for residues 73 - 88 are valid. Crosslinking at Y91 and D94 bears further investigation, but is not involved in crosslinking to chaperone-subunit complexes, as indicated by immunoblotting with anti-DG antibodies. The loop's function may be to position the tip of the N-terminal domain at the precise height needed to recruit incoming chaperone-subunit complexes.

By using photocrosslinking techniques, specific sites of interactions occurring *in vivo* can be identified. The usher can be crosslinked alone or in the presence of specific pilus subunits to see different combinations of complexes, thereby revealing the interactions made throughout the assembly process. Mass spectrometry can be used to identify the peptides that are bound at specific sites. Since bacterial secretion systems and pilus structures are critical virulence factors, a better understanding of this process can provide information about mechanisms of bacterial pathogenesis and the general processes by which cells secrete proteins across membranes and build organelles. This understanding will facilitate the development of new therapeutic agents that interfere with the chaperone/usher pathway and will provide new treatments for bacterial diseases such as urinary tract infections.

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