Attachment Factor Secretion in Uropathogenic *E. coli* Bacteria

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The purpose of this study was to develop a model for 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. They are composed of monomeric proteins that are assembled and secreted at the cell surface through the chaperone/usher pathway, in which periplasmic chaperones transport pilus subunits to the outer membrane to be secreted through outer membrane usher proteins. The usher PapC in the pyelonephritis-associated pilus gene cluster was hypothesized to function as a twin-pore, dimeric protein, in which the N-terminal target domains of each monomer alternate in recruiting periplasmic chaperones. A fused usher dimer was created through DNA manipulation to disable single domains by mutation. If the domains alternate in recruiting chaperones, a single disabled domain will disallow pilus expression. Results show that the current linker sequence used to fuse the usher monomers leads to protein degradation, requiring a new construct for effective study of N-terminal mutations. 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

All cells must control the movement of molecules into and out of the cell. This includes proteins that are secreted for a variety of functions. Bacterial protein secretion has a strong relation to pathogenesis, necessitating an understanding of molecular secretion mechanisms. Proteins to be secreted in Gram-negative bacteria must cross the inner membrane (IM), periplasm, cell wall, and outer membrane (OM). For a common group of adhesive extracellular organelles, this is carried out by the chaperone/usher (CU) pathway (Thanassi et al., 1998a). These fibrous surface organelles, known as pili, are key factors in host cell recognition and attachment. Uropathogenic *Escherichia coli* uses the CU pathway to assemble pili known as P pili, encoded by the *pap* gene cluster (Fig. 1). P pili are responsible for cellular colonization in pyelonephritis, an infection of the kidney (Roberts et al., 1994). The P pilus consists of a tip fibrillum connected to a helical rod (Fig. 1) (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 OM usher (PapC) (Thanassi et al., 1998a). All proteins, for both structure and assembly, cross the IM into the periplasm through the Sec secretory pathway (Fig. 2) (Pugsley, 1993). Pilus structural subunits then interact with the chaperone

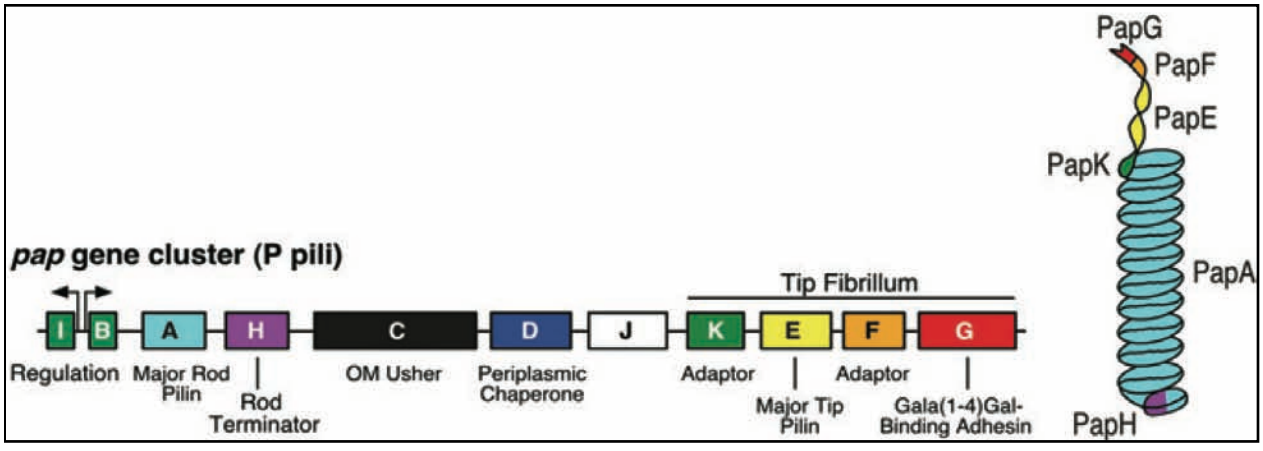

Fig. 1. P pilus gene cluster with structural organization of pilus (shown at right) (So & Thanassi, 2006).

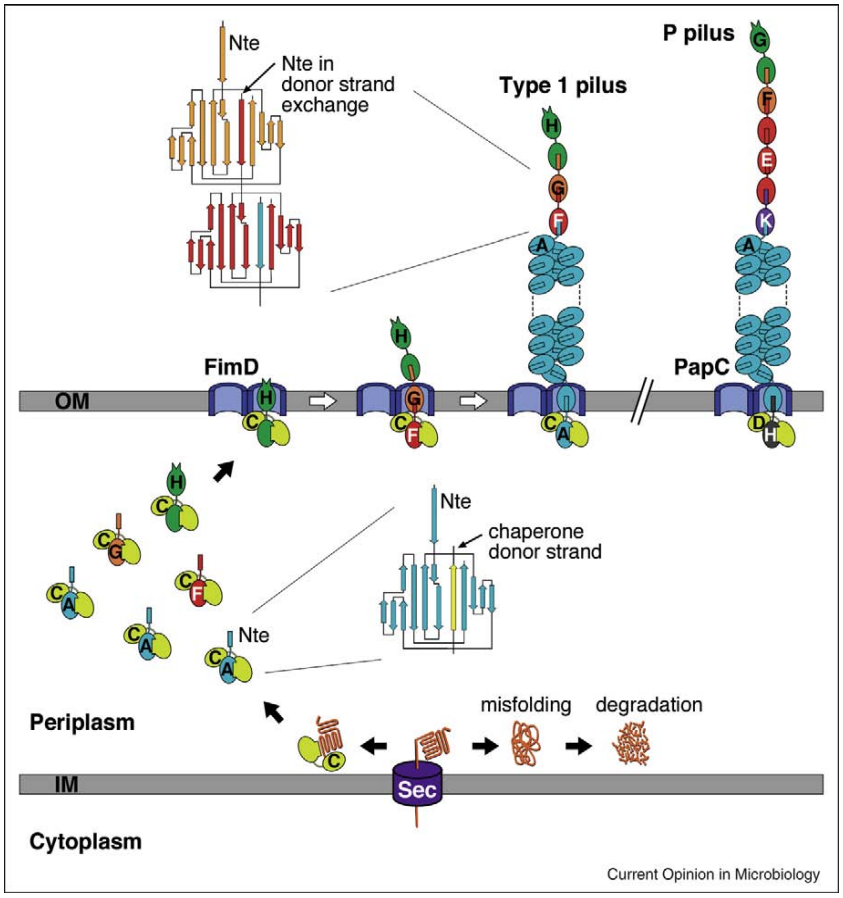

Fig. 2. Model for pilus biogenesis by the CU pathway.
Donor-strand complementation and donor-strand exchange are shown for assembly and secretion of the Type 1 pilus, which is analagous in structure and assembly to the P pilus. Proteins are indicated by letter (A for PapA, G for PapG, etc.). The assembled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

(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 fiber biogenesis (Sauer et al., 2002; Zavialov et al., 2005).

Although much is known about chaperone-subunit and subunit-subunit interactions, information still remains to be uncovered about the interactions regarding the OM usher. The OM usher functions as an asymmetric twin-pore dimer, with pilus subunits being assembled through only one of the pores (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 (So & Thanassi, 2006). A plug domain prevents unintended secretion of pilus subunit proteins (Remaut et al., 2008).

A recent study by Remaut et al. (2008) of an analogous pilus assembly system using cryo-electron microscopy imaging suggested that the N-terminal domains of both ushers are used in recruiting chaperone-subunit complexes. From this model of both N-terminal domains of the dimeric usher being necessary for pilus production, a hypothesis was formed of alternating N-terminal domains in chaperone-subunit recruitment (Fig. 3) (Remaut et al., 2008). In my study, I examined the current model though the creation of a fused usher dimer, with the aim of providing genetic evidence for the N-terminal domains of both ushers being necessary for pilus biogenesis. A plasmid containing two copies of the OM usher linked by a protein chain was created, and the N-terminal domain of one usher was subsequently disabled. Pilus and usher expression were then assayed. Thus far experiments have not proven the hypothesis, due to degradation of the linked usher. A solution to this is currently being investigated.

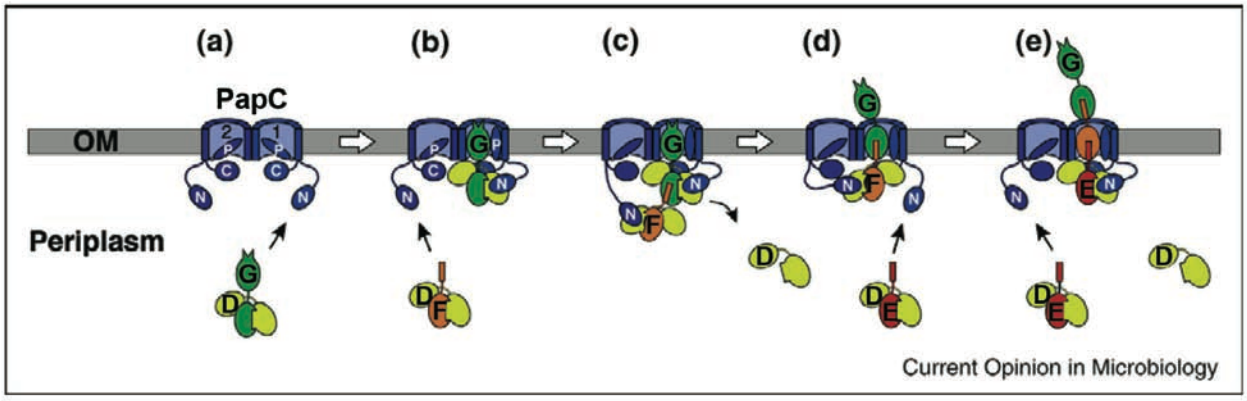

Fig. 3. Model for P pilus assembly and seretion at the OM usher.
(a) The PapC dimer is shown, with the ushers labeled 1 and 2. The N-terminal, C-terminal, and plug domains of each usher are labeled N, C, and P, respectively. **(b)** Recruitment of the first chaperone-subunit complex by usher 1 opens its plug domain, allowing protein secretion. **(b-e)** N-terminal domains alternate in recruting periplasmic chaperone-subunit complexes (modified from Li & Thanassi, 2009).

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 5ml Luria-Bertani (LB) growth medium, with 5µl ampicillin or 15µl tetracycline where appropriate. Colonies were grown on 25µl LB agar plates with 25µl ampicillin or 75µl tetracycline where appropriate. 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 600nm (OD_{600}) before inducing. Plasmids with arabinose-inducible promoters were induced with 0.1% L-arabinose and those with isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoters were induced with 0.1mM IPTG, both for 1 hour.

The mutant p1NK was 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 resistant (Amp^r). C-terminal

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)
AAEC185	Δ*fim* (lacking entire *fim* gene cluster)	Blomfield et al. (1991)
Plasmids		
pMON6235Δcat	vector, P_{ara}, Amp^r	Jones et al. (1997)
pMJ2	Δ*papC pap* operon in vector pACYC184, P_{trc}, Tet^r	Thanassi et al. (1998b)
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)
pGEM/link	PapC with linker and restriction sites in pGEM-T Easy	This study
p1NK	KPN1 and NHE1 restriction sites inserted at C-terminal of pDG2	This study
pEP2C	Two linked PapC in pDG2	This study
pEP1F3A	PapC F3A in first PapC of pEP2C	This study

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

Table 2. Primers used in this study

Primer	Sequence[a] (5'-3')	Reference[b]
papCF3A	GCCAGTGCCGTTGAGGCTAATACAGATGTACTTGACGC	Ng et al. (2004)
NHE1KPN1	CGCCTCAGAAAGCTAGCGCGGGTACCCTGGTCCCCCGGGGC	
linkF[c]	GCTAGCGGTGGCGGTGGCTCTGGTGGCGGTGGCTCTGTTGAGTTTAATACAGA	
linkR	GGTACCTTTCTGAGGCGTACAGG	

(a) Primers with no reverse indicated were used for mutagenesis, and as such have a reverse primer that is the reverse complement of the foward primer
(b) If no reference is given, primer is from this study.
(c) Inclusion of a flexible linker (GGGGSGGGGS, underlined)

NHE1 and KPN1 restriction sites were inserted using a QuikChange site-directed mutagenesis kit (Stratagene) and DH5α as the host strain. The primers used for mutagenesis and polymerase chain reaction (PCR) of specific segments are listed in Table 2. All mutants were completely sequenced to ensure the presence of the desired mutation and the absence of unintended mutations.

To construct pGEM/link, oligonucleotides (primers) specific to PapC in pDG2 from Invitrogen (Invitrogen Corporation) were used. An NHE1 restriction site and flexible linker were included in the forward primer, and a KPN1 restriction site was included in the reverse primer. The purified synthetic PapC/link was cleaned using ethanol purification as described (Sambrook et al., 1989) and cloned into pGEM- T Easy vector (Promega) using a Quick Ligation Kit (New England BioLabs (NEB), Inc.). The pGEM/link plasmid was then transformed into chemically competent DH5α cell, using bromo-chloro-indolyl-galactopyranoside (X-gal)/ IPTG blue-white screening to select colonies for examination. Cells were spread on ampicillin LB agar plates, so untransformed cells could not grow. Selected colonies were then restreaked and their plasmids purified using a Promega Wizard® Plus Miniprep kit (Promega). Correct plasmid construction was then checked first by gel electrophoresis of digestion products with NHE1 and KPN1 enzymes, then by completely sequencing the relevant area.

The vector p1NK and insert-containing plasmid pGEM/link were then digested completely with NHE1 and KPN1 and run through an 0.8% agarose gel stained with ethidium bromide (Caution: ethidium bromide is a possible carcinogen, great care was taken to minimize exposure when handling). Fragments to be ligated were cut out of the gel without exposure to ultraviolet light, as UV exposure has been shown to lead to ineffective ligation (Gründemann & Schömig, 1996), and cleaned with a QIAquick Gel Extraction Kit (Qiagen). PapC/link insert was then ligated into p1NK using a Quick Ligation Kit (NEB) and transformed into DH5α using ampicillin resistance for selection to create plasmid pEP2C. Plasmid DNA was then extracted and checked by agarose gel analysis of restriction/digestion.

The plasmid pEP1F3A was derived from pEP2C by introducing a mutation at the N-terminus of the first usher using a QuikChange site-directed mutagenesis kit. This mutation involved an alanine substitution at the third N-terminal amino acid, and had been previously shown to effective disable the usher N-terminal domain (as pili were not expressed), while leaving structural integrity unaffected (Ng et al., 2004). Mutation of only the first usher was confirmed through sequencing.

Outer Membrane Isolation

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 (Baneyx and Georgiou, 1990).

Haemagglutination Assay

Haemagglutination assays were performed as described (Thanassi et al., 2002) using *E. coli* host strain AAEC185, which lacks the *fim* gene cluster. Plasmid pMJ2, lacking PapC, was

transformed into the cells along with pMON6235Δcat vector, pMJ3 WT PapC, pEP2C, or pEP1F3A. Bacterial samples were serially diluted in microtitre plates to test the ability of the plasmid to produce P pili that could agglutinate human erythrocytes.

RESULTS

To investigate the function of a single N-terminus in the usher dimer, a fused usher dimer was constructed. To do this, a second PapC usher gene was inserted into a plasmid containing a single copy of the PapC gene, along with a flexible linker connecting the two ushers. At all stages of plasmid development, proper construction 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)). Difficulty was encountered in ligating the insert that was released from the intermediate pGEM plasmid through enzymatic digestion. While electrophoresis gels showed the presence of proper length products (Fig. 4), no transformed colonies grew on agar plates. As the damaging effects of UV radiation on DNA ligation efficiency had been previously described (Gründemann & Schömig, 1996), I hypothesized that UV radiation from either photographic equipment or the UV light box used to

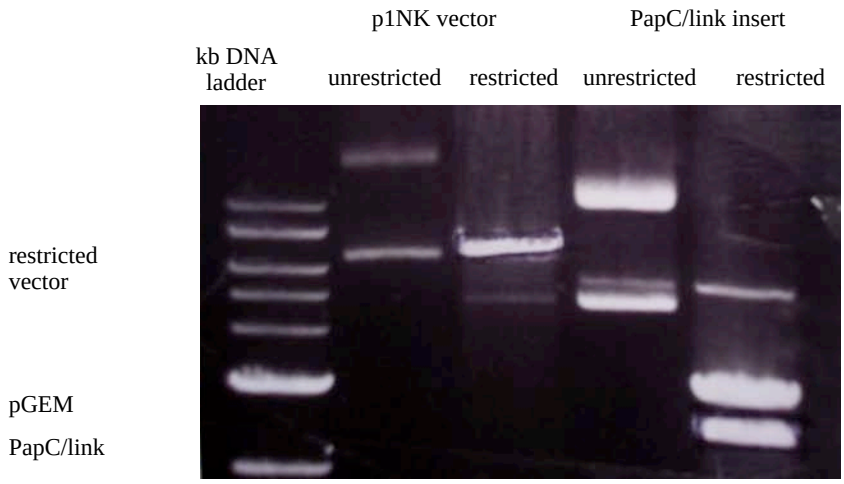

Fig. 4. Restricted DNA fragments separated by gel electrophoresis. Bands from this image were cut out and purified.

view DNA while removing it from the agarose gel was damaging the DNA. To remedy this, a smaller sample of DNA was run alongside the lane to be used for ligation. Lanes containing the larger DNA sample were removed and set aside before exposing the gel to UV radiation. The gel that now contained only the smaller samples of DNA was then photographed as normal, and tick marks made on the gel to identify DNA position. Removed lanes were then placed back into their original positions, and tick marks on the UV-exposed lanes were used as reference points for removing the DNA from the correct lanes. This method led to the presence of colonies on transformed plates.

Alanine substitution mutagenesis was then performed on the first usher (usher 1) of the double usher plasmid to disable its N-terminus. Sequencing this mutated plasmid, called pEP1F3A, posed a difficulty in that currently existing sequencing primers that began within mature PapC would bind to that location in both usher genes. This was beneficial, however, in showing that mutants still contained two copies of the usher. With two locations for the primer to bind, sequenced DNA contained a double usher showed interfering DNA base signals when sequenced with a C-terminal primer, as the reading frame extended beyond the identical segments of DNA (data not shown). To ensure that the mutation occurred in only the first usher, a sequencing primer was designed that binds to the link sequence between the two ushers. This primer was able to sequence the N-terminal domain of the second usher effectively (data not shown).

Alanine substitution of the F3 residue of the PapC usher had been previously shown to disallow pilus assembly when coexpressed with a Δ*papC pap* operon (Ng et al., 2004). However, results of a haemagglutination assay showed that the fused usher mutant with a disabled usher 1

Table 3. Haemagglutination analysis (HA) of PapC mutants

PapC	HA titre[a]
Vector	0
WT	32
pEP2C	16
pEP1F3A	16

(a) HA titre is the highest fold dilution of bacteria still able to agglutinate human red blood cells.

N-terminus was able to produce pili that were effective in agglutinating human erythrocytes (Table 3).

To uncover further information regarding the ability of the fused usher with the disabled N-terminal domain in producing pili, the outer membrane was isolated from bacteria expressing this usher. The outer membrane was prepared and run on an SDS-PAGE gel, which was then blotted with anti-His, an antibody that binds to the C-terminal His-tag (Fig. 5). The wild-type usher showed the strongest monomer band. The duplicated usher showed both dimeric and monomer PapC to be present. The duplicated usher with the F3 alanine substitution mutation showed a strong dimer band, along with a faint monomer band.

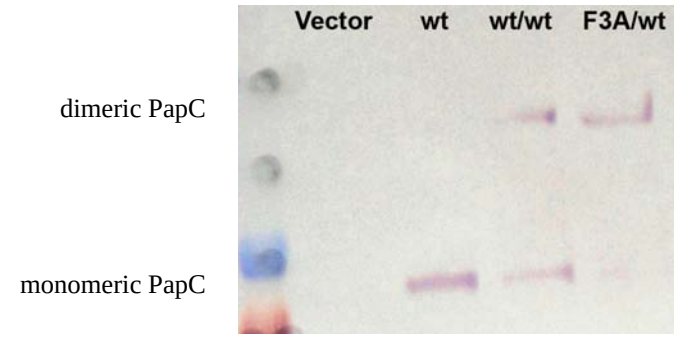

Fig. 5. Expression of PapC by fused usher mutants. SDS-PAGE gel was blotted with anti-His antibody.

The same outer membrane preparations were run again and blotted with anti-PapC to show all PapC present, even that not attached to a His-tag (Fig. 6). When blotted with anti-PapC,

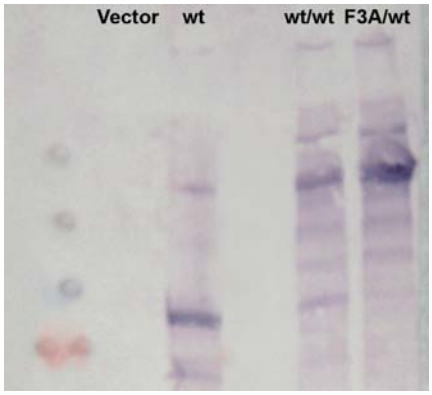

Fig. 6. Expression of PapC by fused usher mutants. SDS-PAGE gel was blotted with anti-PapC antibody.

the gel revealed many bands of varying lengths in the fused usher mutants, showing protein degradation of the fused usher dimer.

DISCUSSION AND CONCLUSIONS

The purpose of this investigation was to create a mutant that facilitates study of the dimeric usher protein, and to use this construct to analyse the function of the usher N-terminal domain. The flexible linker used to fuse the two mature ushers was found to be an ineffective method of creating a fused dimer. According to sequencing results, the double usher construct was created correctly with no mutations that could interfere with expected results (data not shown). Thus, it is the linker itself that is insufficient for attaining desired results.

During initial testing of functional pilus production through haemagglutination analysis, the double usher construct pEP2C behaved as expected, agglutinating human erythrocytes and showing a dimeric band when blotted with anti-His antibodies. However, when the N-terminal domain was disabled and the same assays repeated, the results disagreed with the current model of alternating N termini in the dimeric usher, as the disabled domain unexpectedly produced a functioning pilus.

In an attempt to discover the cause of this discrepancy, OM preparations were blotted with anti-PapC antibody. Due to its nature, anti-PapC does not provide an equal level of clarity in comparison to anti-His, but it has the advantage of binding to all instances of PapC, not only those that have remained attached to their C-terminal His-tag; it shows degraded PapC as well. Blotting with anti-His shows only those proteins that have remained intact, explaining the difference in band strength between the WT and mutant ushers (Fig. 5). When blotted with anti-PapC, the SDS-PAGE gel showed that both dimeric PapC constructs degraded to various sizes, including monomer PapC (Fig. 6). This explains the results of the haemagglutination assay, as PapC was able to function in its WT state, as opposed to functioning as a fused dimer. Thus, it remains unknown if PapC is able to function as a fused dimer.

Alternate methods of a creating a fused usher construct are currently being investigated. Increasing the length of the linker sequence or using a different linker between the two ushers may be effective in stabilizing the dimer form. Constructs with these new linkers will be created and tested as previously described. Another possible approach is to use novel mutations to disallow usher function upon protein degradation. If this method were used, the ushers would not need to be fused; there would only need to be two copies of the PapC gene in a plasmid. If this mutation could be developed, ushers would not be able to assemble pili when degraded to monomers, working only in pairs. This mutation would need to be different from the N-terminal mutation, so that the N-terminal mutation could later be introduced to test N-terminal function.

Although this study did not provide a definitive response to the hypothesized results, it still provided important information for testing the OM usher of the chaperone/usher pathway. While a fused usher dimer may still be feasible, the linker sequence GGGGSGGGGS was found to be

ineffective, as proteins linked using this sequence showed evidence of degradation. Continued study along this direction still has the potential to lead not only to conclusive evidence in support of the current model of usher function, but to an effective method for creating mutations in single monomers of a functional dimer to study the dimeric interaction of individual domains.

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