

THE ROLE OF THE CHAPERONE COOB IN THE FOLDING OF THE CS1 PILUS
SUBUNIT COOA FROM ENTEROTOXIGENIC *ESCHERICHIA COLI*

A Thesis by

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Chemistry.

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*To my dear family and friends
Thank you for all your support*



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ABSTRACT

CS1 pili from enterotoxigenic *Escherichia coli* (ETEC) play an essential role in colonizing host intestinal tissue, leading to severe diarrheal disease. CS1 (coli surface antigen 1) pili are a representative class of pili formed using the alternate chaperone-usher pathway for pilus assembly. For CS1, the assembly of the subunits CooA and the tip CooD requires the chaperone CooB and the outer membrane usher CooC. Despite having little sequence homology, CooB is thought to assist in folding the subunits similarly to known chaperones, such as PapD (for P pili) and FimC (for type I pili), which have been shown to catalyze the folding of pilus subunits. To confirm that CooB functions as a chaperone or catalyst, we monitored the folding of CooA in the presence and absence of CooB. To do this, we generated a mutant of CooA (F84W) and a mutant of CooB (W71F) that allowed us to monitor solely the tryptophan fluorescence of F84WCooA, even in the presence of W71FCooB. The kinetic results indicated that the rate of F84WCooA folding did not increase with W71FCooB concentration, suggesting that W71FCooB acts as a classical chaperone by preventing nonspecific aggregation. Consequently, CooB does not function similarly to PapD and FimC in facilitating the folding of subunits and may represent a form of convergent evolution in how pilus subunits can be assembled.

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LIST OF ABBREVIATIONS

UTI	Urinary Tract Infection
CFA/I	Colonization Factor Antigen I
CS1	Coli Surface Antigen 1
CS2	Coli Surface Antigen 2
CS3	Coli Surface Antigen 3
AFA-III	Afimbrial Adhesins-III
UPEC	Uropathogenic <i>Escherichia Coli</i>
ETEC	Enterotoxigenic <i>Escherichia Coli</i>
HSP	Heat Shock Protein
ATP	Adenosine Triphosphate
CS	Citrate Synthase
DSC	Donor Strand Complementation
DSE	Donor Strand Exchange
CD	Circular Dichroism
MES	2-(N-morpholino)ethanesulfonic acid
SDS PAGE	Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis

CHAPTER 1

INTRODUCTION

1.1 Pathogenic Bacteria

The human body is a complex ecosystem that contains both human and bacterial cells ¹. These bacteria, commonly called the normal microbial flora, inhabit specific regions of the human body, including the skin, mouth, and large intestine ¹. They are not harmful to humans and are essential for normal body functions. While the normal microbial flora play beneficial roles in maintaining health, other bacteria can invade, colonize, and cause infection. These harmful bacteria, called pathogens, possess surface adherence factors, such as pili, which facilitate the colonization of the host and contribute to the development of various diseases, as shown in Table 1².

Table 1

Pathogenic Bacteria Pili and Their Associated Diseases (reproduced from reference ^{2, 22})

Organism	Pili	Morphology	Disease	Reference
<i>E.coli</i>	P	Thick, rigid rods	Pyelonephritis	3, 4
<i>E.coli</i>	S	Thick, rigid rods	UTI	5, 6
<i>E.coli</i>	Type 1	Thick, rigid rods	Cystitis	7, 8
<i>B.pertussis</i>	Type 1	Thick, rigid rods	Whooping cough	9, 10
<i>E.coli</i>	CFA/I	Thick, rigid rods	Diarrhea	11
<i>E.coli</i>	CS1	Thick, rigid rods	Diarrhea	12-14
<i>E.coli</i>	CS2	Thick, rigid rods	Diarrhea	15, 16
<i>K.pneumoniae</i>	Type 3	Thin, flexible rods	Pneumonia	17
<i>E.coli</i>	CS3	Thin, flexible fibrillae	Diarrhea	18, 19
<i>E.coli</i>	AFA-III	Thin, flexible fibrillae	Diarrhea	20, 21

1.2 The Role of Pili in Pathogenic Bacteria

For bacteria to colonize and cause an infection, they must first attach to the host, making bacterial adhesion a critical initial step in the pathogenesis of various infectious diseases²³. The net repulsive force, resulting from the negative charges present on both the bacterial and host cells, prevents bacterial attachment to the host²³. To overcome this problem, most pathogenic gram-negative bacteria have evolved non-flagellar, hair-like surface structures called pili (also known as fimbriae), enabling them to attach to host cells without coming into close proximity, thus avoiding the strong repulsive force²³.

Pili are long proteinaceous polymers primarily composed of repeating pilin subunits²⁴. These pili have surface-exposed adhesins located at their tips, which function as carbohydrate-binding proteins (lectins), recognizing oligosaccharide chains attached to glycoprotein and glycolipid receptors on host cells^{23, 25}. Adhesin binding is receptor-specific; each adhesin type recognizes and specifically binds to host cells expressing particular receptors^{25, 26}. For example, both P pili and type 1 pili are essential virulence factors for uropathogenic *Escherichia coli* (UPEC) which cause urinary tract infections, including pyelonephritis and cystitis²⁷. However, their respective adhesins exhibit distinct receptor-binding specificities. The P pili adhesin, PapG, binds to galactosyl- α (1-4)-galactosyl-containing receptors located on the kidney epithelium, whereas the type 1 pili adhesin, FimH, binds to mannosylated receptors present on the bladder epithelium²⁸.

Pili represents a crucial target for vaccine development due to their pivotal role in bacterial adhesion and colonization²³. A vaccine designed to block pili binding to host receptors could effectively prevent the establishment of infections caused by pathogenic bacteria²⁴.

1.3 Pili Morphology

The pili of pathogenic bacteria can be subdivided into four major groups based on their morphological characteristics (Figure 1), which include thick rigid rods, thin flexible rods, thin flexible fibrillae, and bundle-forming rods.^{2, 22, 27}. The thick, rigid rods with axial holes (Figure 1A) have a diameter ranging from 2 to 10 nm and are evenly distributed around the bacterial cell surface^{2, 29}. These rods consist of thinner tip rods that attach to their distal ends, creating a composite structure for the pilus². The thin pilus rods (Figure 1B), measuring 2-3 nm in diameter, exhibit greater flexibility than rigid rods and lack axial holes^{2, 30}. Even thinner fibers, referred to as thin fibrillae (Figure 1C), are very thin, flexible fibers measuring less than 2 nm in diameter, and they tend to coil into an amorphous mass on the bacterial surface². Bundle-forming pili (Figure 1D) are polar fimbriae that aggregate laterally, creating large rope-like structures^{27, 29}.

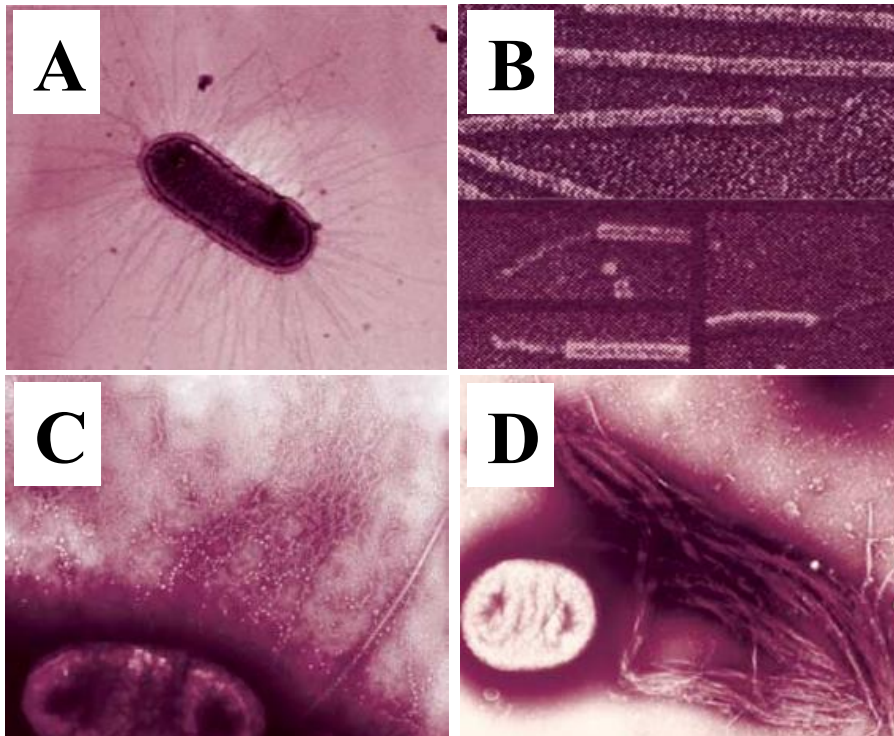


Figure 1. Pili morphologies of pathogenic bacteria (reproduced from reference²⁷). (A) Thick, rigid rods (2-10 nm in diameter). (B) P pili of UPEC exhibit thin, flexible tip rods (2-3 nm) attached to the ends of thick, rigid rods (2-10 nm). (C) very thin, flexible fibrillae (< 2 nm). (D) Bundle-forming flexible rods.

1.4 Molecular Chaperones in Protein Folding

Protein folding is the process by which a polypeptide chain, composed of a specific amino acid sequence, folds into a unique three-dimensional (3D) structure³¹. This 3D structure, known as the native state, is crucial for the protein to perform its biological function³². According to Anfinsen's principle of protein folding, the amino acid sequence encodes the information necessary for a protein to fold into its native conformation³³. Failure of proteins to fold into their native 3D structure can lead to aggregation. The accumulation of aggregates is associated with neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease³⁴. Therefore, 'folding helpers' are crucial in ensuring that proteins fold correctly to prevent aggregation.

Molecular chaperones serve as these 'folding helpers' by binding to unfolded or partially folded proteins, thus protecting them from aggregation³⁵. These chaperones belong to the group of heat shock proteins (HSPs), which are produced in both bacteria and eukaryotes in response to various stressful conditions, such as extreme heat or low oxygen levels (hypoxia)^{36,37}. Among the different types of HSPs, the HSP60, HSP70, and HSP90 families are the most extensively studied HSPs that function as chaperones, facilitate protein folding in an ATP-dependent manner³⁷.

The GroE chaperone system, consisting of GroEL and its co-chaperone GroES, is the bacterial homolog of HSP60 and HSP10 encoded by the *groE* operon³⁵. These chaperones, found in *Escherichia coli*, were among the earliest to be studied in detail to understand their role in protein folding^{35,38}. The GroEL-GroES folding machinery comprises two GroEL heptameric rings forming a complex, along with a GroES heptamer ring acting as a cap binding to the GroEL³⁷. In the first step of the folding process, GroEL binds to the unfolded protein, preventing aggregation³⁸. Once the co-chaperone GroES and ATP bind to GroEL, the unfolded protein is then released into the central chamber of GroEL³⁷⁻³⁹. Within this protected environment, the protein can fold

correctly utilizing energy from ATP hydrolysis without interference from the surrounding environment³⁷⁻³⁹. Subsequently, after ATP hydrolysis and discharge of GroES, the folded protein is released from the GroEL chamber (Figure 2)³⁷⁻³⁹.

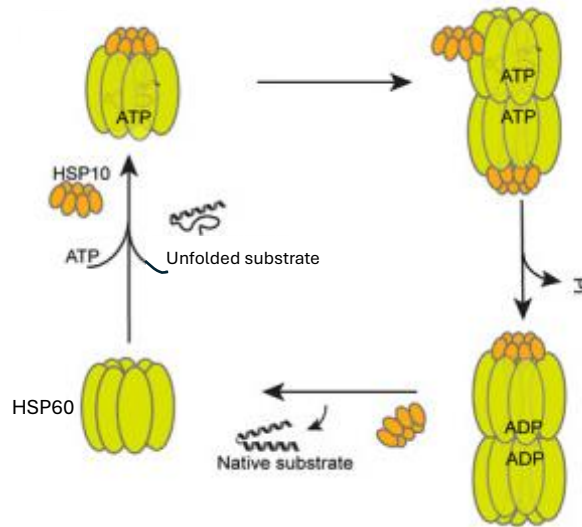


Figure 2. HSP60-HSP10 (GroEL-GroES) chaperone cycle model (reproduced from reference³⁷).

The effect of chaperone GroE on protein folding was studied by using the refolding of citrate synthase (CS) as a model system because its unfolding is irreversible due to rapid aggregation during refolding³⁵. However, in the presence of GroE and ATP, there is an increase in the yield of active, folded CS while simultaneously reducing the formation of CS aggregates³⁵. In addition, the rate of formation of active CS did not change with increasing GroE – only the yield³⁵. This suggests that GroE acts as a chaperone, binding to the folding intermediates of CS during refolding, thereby preventing aggregation and enabling correct folding³⁵.

Unlike intracellular ATP-dependent molecular chaperones such as GroE, which require ATP as an energy source during protein folding, the chaperones involved in pilus assembly system function in an ATP-independent manner²⁵. The binding of chaperones to pilus subunits has been

shown to protect them from proteolytic degradation in the periplasm, prevent premature polymerization and aggregation of pilus subunits, and facilitate the transport of pilus subunits to the outer membrane usher, which serves as the assembly platform for the pilus ^{25,40}.

1.5 Pilus Assembly Pathway

1.5.1 Chaperone-Usher Pathway

Pathogenic gram-negative bacteria utilize various pilus assembly pathways for pili formation. Among these pathways, the chaperone-usher pathway is the most extensively studied mechanism for assembling the majority of pili, such as type 1 and P pili of uropathogenic *Escherichia coli* (UPEC), which causes cystitis and pyelonephritis ⁴¹. This pathway requires a periplasmic chaperone that facilitates pilus subunit folding and prevents subunit aggregation, along with an outer-membrane usher that serves as an assembly platform ⁴⁰. Pilus subunits are transported across the bacterial inner membrane into the periplasm via the general secretory pathway (SecYEG translocon) ⁴². In the absence of chaperones, pilus subunits are unstable and fail to fold correctly due to an incomplete immunoglobulin-like (Ig-like) fold, containing only six β -strands and lacking the seventh C-terminal G β -strand ⁴². The absence of this strand results in the formation of a deep hydrophobic groove on the pilus subunit surface with five hydrophobic pockets, known as P1 to P5 pockets ⁴².

The periplasmic chaperones of the chaperone-usher pathway are 25 kDa proteins with two Ig-like domains arranged in a 'boomerang' shape ^{40, 43, 44}. These chaperones contain highly conserved residues that are crucial to their function ². Mutations in the highly conserved residues of the P pilus chaperone PapD, specifically Lys-112 and Arg-8, lead to the complete loss of chaperone function and abolish pilus biogenesis ^{2,44}.

Chaperones facilitate pilus subunits folding through a process called donor strand complementation^{44,45}. In this process, the chaperone donates its own G β -strand, which contains four alternating hydrophobic residues named P1 to P4, to the P1 to P4 hydrophobic pockets in the subunit groove, forming stable chaperone-subunit complexes^{40, 45}. The P5 pocket remains accessible, allowing interaction with an incoming subunit at the outer membrane usher for polymerization (Figure 3A)⁴⁵. In a typical Ig-fold, the orientation of the seventh G strand is antiparallel to the F strand⁴⁵. However, in the chaperone-complemented Ig-fold of the subunit, the G strand donated by the chaperone runs parallel to the subunit F strand, making the chaperone-complemented Ig-fold atypical (Figure 3B)⁴⁵.

The chaperone-subunit complexes are targeted to outer membrane (OM) ushers for pilus assembly. The OM ushers are 90 kDa pore-forming proteins composed of four domains: a large outer-membrane-spanning beta-barrel domain (approximately 500 residues), an N-terminal periplasmic domain (around 125 residues), a C-terminal periplasmic domain (approximately 170 residues), and a plug domain (about 110 residues)^{40,45}. The primary function of these OM ushers is to catalyze the ordered polymerization of pilus subunits and translocate the growing pilus across the outer membrane⁴⁵.

All pilus subunits contain a 10-20 residue N-terminal extension (Nte) that is not part of the subunit Ig-like fold, but essential for subunit-subunit interaction in donor strand exchange (DSE)^{40,45}. During DSE, the chaperone G donor strand from the last assembled pilus subunit is replaced by the Nte of the subsequent subunit (Figure 4A, B)⁴⁰. The Nte of the subunit contains four hydrophobic residues, designated as P2 to P5 residues⁴⁵. These residues progressively occupy the hydrophobic pockets in the groove of the previous subunit, starting with the insertion of the P5 residue into the P5 pocket, which serves as the initiation site for DSE^{40,45}. Subsequently, the P4,

P3, and P2 residues are sequentially inserted into their respective P4, P3, and P2 pockets ⁴⁰. When these hydrophobic pockets are occupied by their corresponding hydrophobic residues of the Nte, the chaperone G strand is displaced, allowing the chaperone to be released into the periplasm ⁴⁰. The proposed mechanism for this process is called the ‘zip-in, zip-out’ mechanism (Figure 5), where the Nte of the incoming subunit progressively displaces the G donor strand from the previously assembled subunit ^{40, 45}. This exchange between the G donor strand and Nte repeats until a long polymer pilus forms, with all the pilus subunits connected by their Ntes ⁴⁵.

After DSE, the orientation of the G strand changes from parallel to the F strand when it is donated by the chaperone, to antiparallel when donated by the Nte of the incoming subunit, resulting in the reconstitution of a typical Ig fold (Figure 4C) ^{44, 46}. This change in G strand orientation is accompanied by a conformational transition in the subunit, shifting from an expanded state upon binding to the chaperone to a collapsed state upon binding to the Nte of the incoming subunit ⁴⁴. This suggests that the subunit completes its folding only after DSE ⁴⁴.

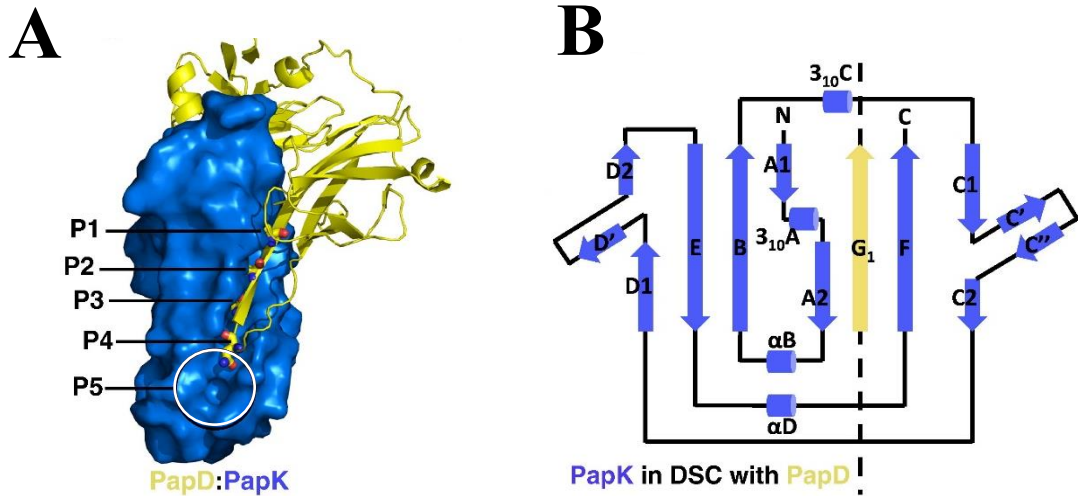


Figure 3. Donor strand complementation (DSC). (A) Crystal structure of P pilus subunit PapK (blue) bound to chaperone PapD (yellow). PapK (blue) is shown as a surface model, and PapD (yellow) is shown as a ribbon model with the hydrophobic residues presented as sticks⁴⁵. The hydrophobic pockets of PapK are labeled as P1-P5, with the P5 pocket indicated by a white open circle⁴⁵. (B) Topology diagram of P pilus subunit PapK (blue) complemented with the G1 strand of chaperone PapD (yellow) (reproduced from reference⁴⁵).

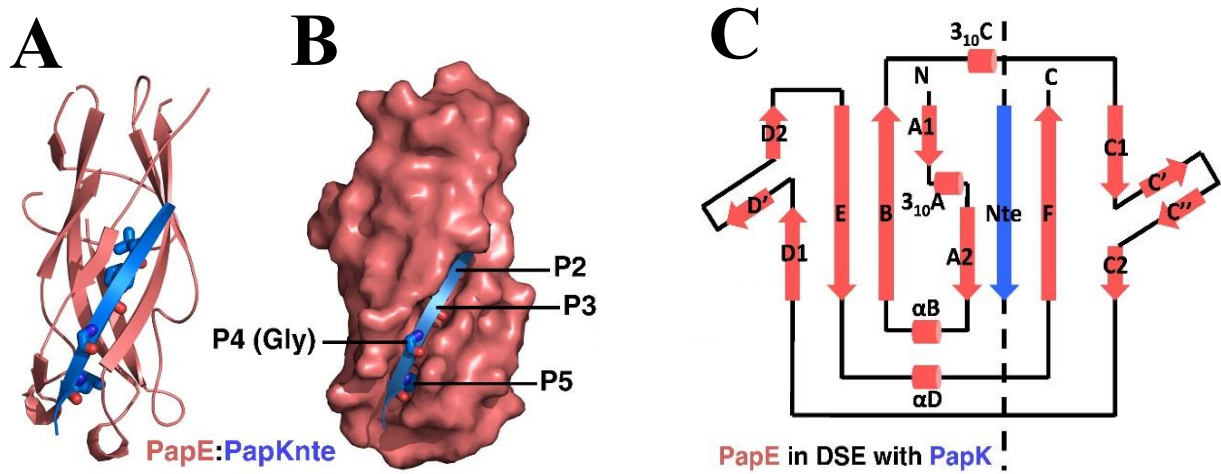


Figure 4. Donor strand exchange (DSE). (A, B) Crystal structures of P pilus subunit PapE (orange) bound to the Nte (blue) of the incoming subunit PapK (reproduced from reference⁴⁵). Pap E is shown as (A) an orange ribbon model and (B) an orange surface model. The Nte of PapK (blue) is presented as a ribbon model in (A) and (B), with the Nte hydrophobic residues P2-P5 shown as sticks⁴⁵. (C) Topology diagram of P pilus subunit PapE (orange) with the Nte of the incoming subunit PapK (blue) (reproduced from reference⁴⁵).

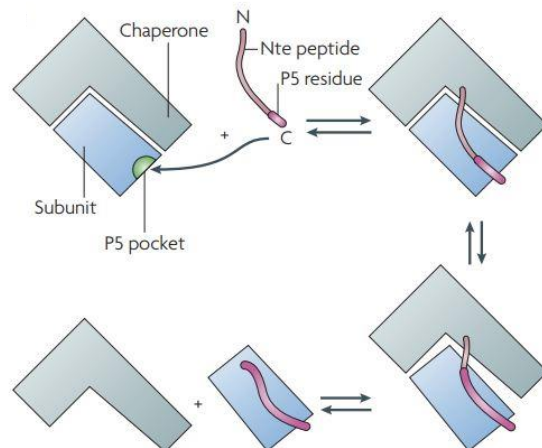


Figure 5. The donor strand exchange ‘zip-in, zip-out’ mechanism (reproduced from reference ⁴⁰). The chaperone G donor strand is progressively displaced by the Nte of the incoming subunit (shown in pink). This process is initiated when the Nte P5 residue is inserted into the P5 pocket of the previously assembled subunit ⁴⁰. After displacing the G donor strand, the chaperone is released from the subunit.

1.5.2 Alternate chaperone-usher pathway

The class 5 pili, which include colonization factor I (CFA/I), coli surface antigen (CS)1, CS2, CS14, CS17, and CS19, are classified as the alternate chaperone-usher pathway pili ²³. This group of pili is associated with diarrheal diseases caused by enterotoxigenic *Escherichia coli* (ETEC) ⁴⁷. Class 5 pili are assembled through the alternate chaperone-usher pathway, distinguishing them from the classical chaperone-usher pathway utilized for assembling P pili and type 1 pili of uropathogenic *Escherichia coli* (UPEC) ⁴⁸.

Although the structural and assembly proteins in this pathway do not share apparent sequence similarity to those of the classical chaperone-usher pathway, the requirement for a periplasmic chaperone and outer membrane usher in the biogenesis of both the alternate and classical chaperone-usher pili suggests that these two types of pili may be assembled through a similar mechanism termed donor strand complementation and exchange (Table 2) ^{24, 49}.

In both assembly pathways, the pilin subunits, after being secreted into the periplasm via the SecYEG translocon, form complexes with chaperones to prevent proteolytic degradation^{40,49}. The chaperones stabilize and complete the Ig-like fold of the subunits, which lack a seventh G β -strand, by donating their G β -strand through donor strand complementation⁴⁴. Subsequently, these subunit-chaperone complexes are targeted to the outer membrane usher for assembly into pili. During assembly, a process called donor strand exchange occurs, in which the chaperone G donor strand from the previously assembled subunit is replaced by the N-terminal extension (Nte) of the incoming subunit⁴⁰ (For more details about donor strand complementation and exchange, refer to Chapter 1.5.1 chaperone-usher pathway). The growing pili are then secreted to the bacterial cell surface through the outer membrane usher pore⁴².

Periplasmic chaperones involved in pili assembly contain conserved residues that play a critical role in pilin subunit binding and folding during donor strand complementation². Chaperones in both assembly pathways, the classical chaperone-usher (CU) and the alternate CU show low sequence similarity (Figure 6). This suggests that chaperones within each pathway may possess distinct sets of functionally important residues.

Previous studies comparing primary structures of chaperones from the classical CU pathway using multiple sequence alignment (MSA) have identified several highly conserved residues critical to chaperone function². These include Arg-8 and Lys-112, located in the cleft of the PapD chaperone, forming the initial interaction site between the chaperone and the pilin subunit (Figure 8)^{2,44}. Mutations in Arg-8 and Lys-112 result in a complete loss of chaperone function, preventing binding to the pilin subunit and thus abrogating pili assembly^{2,40,44}.

We have conducted a more recent sequence alignment of chaperones from both the classical CU and alternate CU pathways. Consistent with previous findings, the MSA results show

more highly conserved residues in the classical CU pathway chaperone sequences. In contrast, the MSA of chaperones in the alternate CU pathway shows a significantly lower number of highly conserved residues. Additionally, chaperones in the alternate CU pathway lack the invariant residues Arg-8 and Lys-112, which have been shown to be crucial for pilin subunit binding and folding in the classical CU pathway ⁴⁴.

Despite this, functionally important residues in the chaperones of the alternate CU pathway can be identified through CoeViz, a web-based tool designed for analyzing and visualizing the coevolution of protein residues ⁵⁰. CoeViz utilizes evolutionary information derived from MSA to identify coevolving residues that are likely to be in close spatial proximity within the folded protein structure which are involved in the same function ⁵⁰. Consequently, changes in one residue often lead to changes in another residue. The coevolution scores generated by CoeViz indicate the strength of the coevolutionary relationship between residues ⁵⁰. High scores (greater than 0.3) suggest strong coevolution, while low scores (less than 0.3) represent weak or no covariation ⁵⁰.

A pair of coevolving residues, Tyr-182 and Leu-123, in CfaA (a chaperone of the alternate CU pathway) has been visualized using the CoeViz hierarchical cluster tree (Figure 7) ⁵⁰. These residues exhibit a high coevolution score of 0.37 ⁵⁰. When mapped onto the 3D structure of the chaperone CfaA bound to the pilin subunit CfaB, we observe that Tyr-182 and Leu-123 are located in the subunit binding cleft region of the chaperone and closely interact with the C-terminus of the pilin subunit (Figure 9). This suggests that both residues may be necessary for the chaperone to form the initial interactive surfaces that contact the pilin subunit for binding and folding.

```

sp|P15319|PapD      -AVSLDRTRAVFDGSEKSMTLDISNDNKQLPYLAQ---AWIENEN-----QEKIITGPV      50
sp|P25732|CfaA     NFMIYPISKDLKNGNSEL--VRVYKSKEIQYIKIYTKKIINPGTTEEYKVDIPNWDGGL      58
      :   : : : * . : : : : . . . * : * : * : . : * :
      :

sp|P15319|PapD     IATPPVQRLEPGAKSMVRLSTTPDISKLPQDRESLFYFNLREIPRSEKANVLQIALQT-      109
sp|P25732|CfaA     VVTPQKVILPAGASKSIRLTQF----KIPKKE-EVYRVYFEAVKPDSKENVIDNKLLTTE      113
      : . * * * * * * . . . * * : * * : : : * *
      :

sp|P15319|PapD     -KIKLFYRPAAIKTRPNEVWQDQLILNKVSGGYRIENPTPYVTVIGLGGSEKQAEEGEF      168
sp|P25732|CfaA     LSVNIIYAA-LIRSLPSEQNI---SLN-I-----                        137
      . : : : * * : : * * * * :

sp|P15319|PapD     ETVMLSPRSEQTVKSANYNTPYLSYINDY-GGRPVLVSLFCNGSR----CSVKKEK-----      218
sp|P25732|CfaA     -----SRNAKKNIIYNNGNVRAGVKDIYFCKSSNIDDNCVKAYNKNIYP      183
      * * : . : * * . . : : * . * * * :

sp|P15319|PapD     ----      218
sp|P25732|CfaA     EKVI      187

```

Figure 6. Amino acid sequence alignment of chaperones from the classical chaperone-usher pathway (PapD) and the alternate chaperone-usher pathway (CfaA). ClustalOmega was used to align the sequences⁵¹. The sequence identity between PapD and CfaA is 19.88%⁵¹. Identical residues are indicated with an asterisk (*), strongly similar residues with a colon (:), and weakly similar residues with a period (.).

Table 2.

Chaperone-Usher and Alternate Chaperone-Usher Pathway Pili (reproduced from reference^{15, 52})

Chaperone-Usher Pathway Assembled Pili					
Pili	Organism	Chaperone	Usher	Disease	Reference
P	<i>E.coli</i>	PapD	PapC	Pyelonephritis	23, 44
Type 1	<i>E.coli</i>	FimC	FimD	Cystitis	52
S	<i>E.coli</i>	SfaE	SfaF	UTI	5, 53
Type 2 and 3	<i>B.pertussis</i>	FimB	FimC	Whooping cough	10, 54, 55
AFA-III	<i>E.coli</i>	AfaB	AfaC	Diarrhea	21, 56
Alternate Chaperone-Usher Pathway Assembled Pili					
Pili	Organism	Chaperone	Usher	Disease	Reference
CFA/I	<i>E.coli</i>	CfaA	CfaC	Diarrhea	11
CS1	<i>E.coli</i>	CooB	CooC	Diarrhea	57
CS2	<i>E.coli</i>	CotB	CotC	Diarrhea	16
CS17	<i>E.coli</i>	CsbB	CsbC	Diarrhea	58

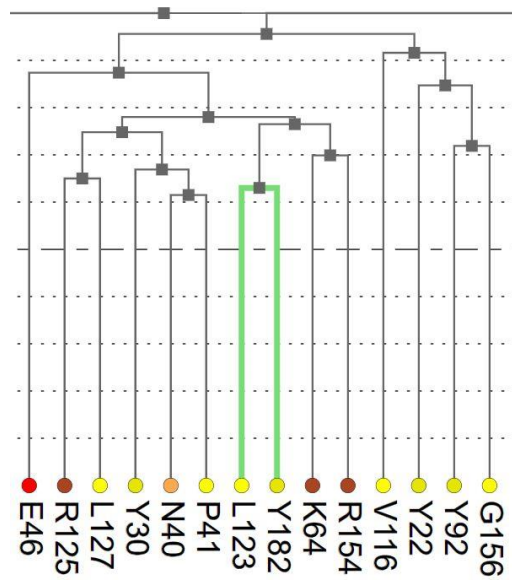


Figure 7. A fragment of the CoeViz hierarchical cluster tree shows the coevolving residues Tyr-182 and Leu-123 in chaperone CfaA, highlighted in green (reproduced from reference ⁵⁰). These residues may be important for the binding and folding of pilin subunits.

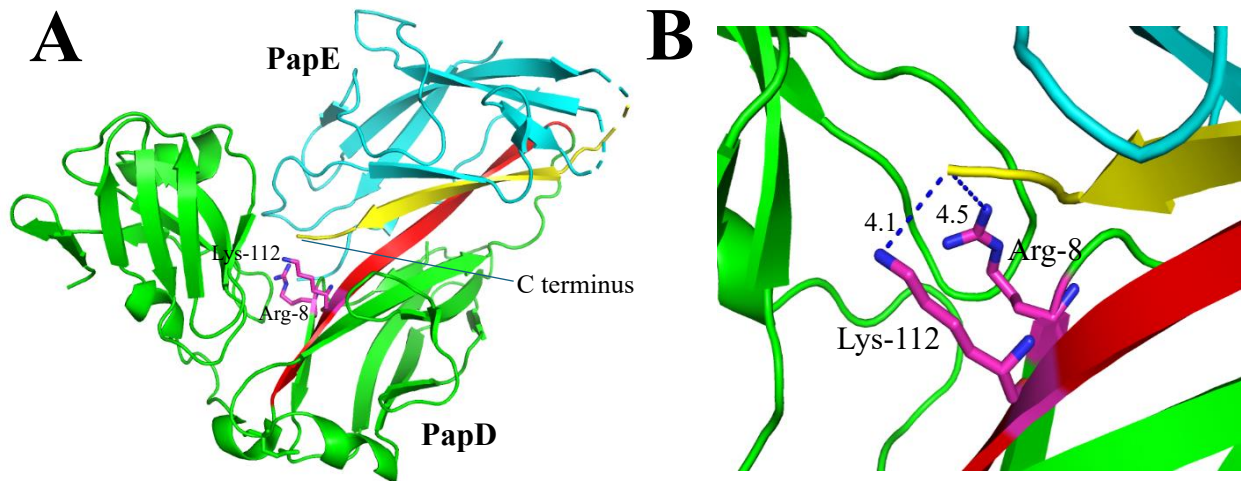


Figure 8. (A) X-ray structure of PapD chaperone bound to PapE pilus subunit (PDB: 1N0L). PapD is shown in green, and PapE is in cyan. The chaperone G strand, which occupies the hydrophobic groove of the subunit, is depicted in red, while the C-terminal strand of the subunit, binding to Arg-8 and Lys-112 in the chaperone cleft, is shown in yellow. (B) A magnified view of the invariant chaperone residues Arg-8 and Lys-112 (shown as sticks) that contact the subunit C terminus. The distances from Arg-8 and Lys-112 to the subunit C terminus are 4.5 Å and 4.1 Å, respectively. The figure was generated using Pymol.

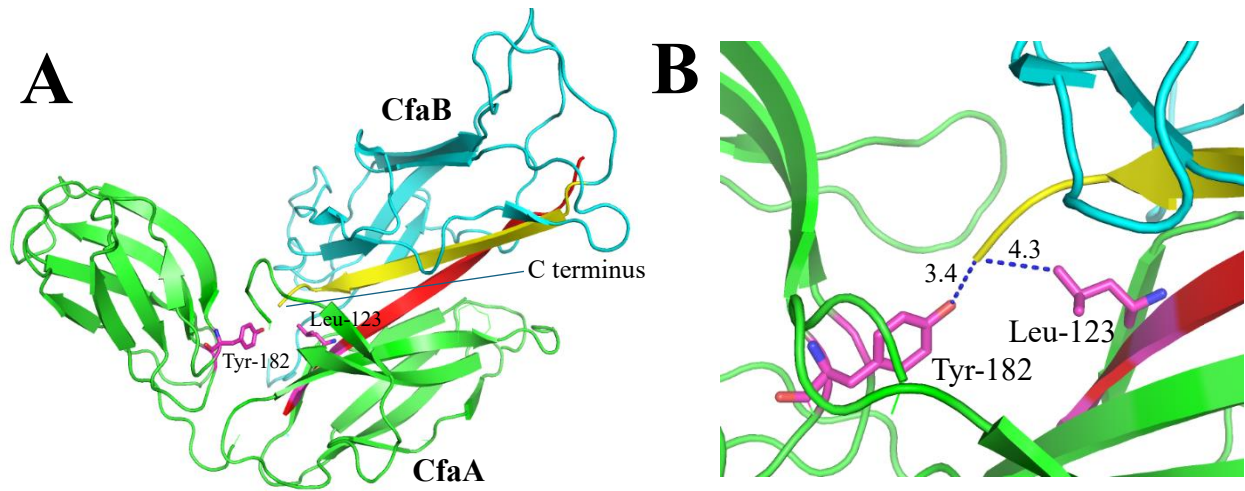


Figure 9. (A) X-ray structure of CfaA chaperone bound to CfaB pilus subunit (PDB: 4Y2O). CfaA is shown in green, and CfaB is in cyan. The chaperone G strand, which occupies the hydrophobic groove of the subunit, is depicted in red, while the C-terminal strand of the subunit, binding to Tyr-182 and Leu-123 in the chaperone cleft, is shown in yellow. (B) A magnified view of the chaperone coevolving residues Tyr-182 and Leu-123 (shown as sticks) that contact the subunit C terminus. The distances from Tyr-182 and Leu-123 to the subunit C terminus are 3.4 Å and 4.3 Å, respectively. The figure was generated using Pymol.

CHAPTER 2

CS1 PILUS ASSEMBLY CHAPERONE COOB FACILITATES THE REFOLDING OF THE CS1 PILUS SUBUNIT COOA BY SUPPRESSING AGGREGATION

2.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) are the primary cause of human diarrheal disease worldwide, especially in developing countries with poor hygiene, sanitation, and limited access to clean water⁴⁸. This disease results in significant mortality among infants and young children⁴⁸. ETEC possess pili on their surface that facilitate the colonization of the human small intestine, which is the critical initial step in ETEC pathogenesis⁴⁷. Therefore, understanding the assembly mechanism of ETEC pili may provide insight into blocking pilus formation, thereby preventing colonization.

The CS1 pili represent a class of ETEC pili assembled by the alternate chaperone-usher pathway. The assembly of CS1 pili requires only four genes, organized in the *coo* operon in the following order: *cooB*, *cooA*, *cooC*, and *cooD*²⁴. The *cooB* gene encodes the 28 kDa chaperone, *cooA* encodes the 15.2 kDa major pilin subunit, *cooC* encodes the 94 kDa outer membrane usher, and *cooD* encodes the 38 kDa tip subunit^{47, 59}. The four protein requirement for pilus assembly contrasts with more complex systems such as Type 1 and Pap, which require 9 and 11 proteins, respectively⁶⁰.

All *Coo* proteins are transported across the cytoplasmic membrane into the periplasm through the Sec-dependent pathway⁴⁷. *CooA* is the major pilin subunit that comprises the body of the CS1 pilus structure²⁴. The pilus tip is associated with the tip subunit, *CooD*, which is required for adherence to host intestinal cells⁴⁸. Although *CooB* is not incorporated into the final pilus structure, it plays an essential role as a chaperone by stabilizing *CooA*, *CooD*, and *CooC* and

preventing their degradation in the periplasm ^{24, 47}. CooC serves as an outer membrane usher, facilitating the assembly and translocation of the pilus across the outer membrane to the cell surface ⁴⁷.

In Figure 10, we present a model for CS1 pilus assembly. The major and tip pilins, CooA and CooD, are secreted into the periplasm via the Sec pathway, where they form complexes with the chaperone CooB ^{47,61}. The CooB-CooD complexes initiate pilus assembly by binding to CooC at the outer membrane ²⁴. CooB is then released and recycled to the periplasm to bind to other unassembled pilins ⁶¹. The assembly process continues as CooB-CooA complexes bind to CooC, displacing CooD and replacing it with CooA ⁵². The repeated addition of CooA at the base of the growing pilus ultimately leads to the extension of the pilus rod across the outer membrane ^{24, 52}.

The role of molecular chaperones, such as GroEL-ES, in protein folding is to prevent the aggregation of nonnative proteins and promote correct folding, thereby increasing the yield of folded proteins ³⁵. Unlike protein-folding enzymes such as disulfide isomerase and prolyl isomerase, molecular chaperones do not accelerate the rate of protein folding ³⁵. However, pilus chaperones involved in pilus assembly via the classical chaperone-usher pathway, such as PapD (for Pap pili) and FimC (for type 1 pili), have been shown to suppress aggregation while catalyzing the folding of pilin subunits ^{44,62}. The pilus chaperone of the alternate chaperone-usher pathway, CooB (for CS1 pili), is thought to assist in folding subunits in the same manner as PapD and FimC, despite the lack of apparent sequence similarity between them (<19% identity) ⁵¹.

To confirm that the pilus chaperone CooB catalyzes the folding of pilin subunits in the CS1 pilus system, we utilized tryptophan fluorescence to monitor the kinetics of refolding of the major pilin subunit, CooA, in the absence and presence of increasing concentrations of CooB. Wild-type CooB contains one tryptophan, whereas wild-type CooA has none. To solely monitor the

tryptophan fluorescence of CooA in the presence of CooB, we generated a mutant of CooA by changing the Phe-84 to tryptophan (F84W) and a mutant of CooB by replacing the Trp-71 with phenylalanine (W71F).

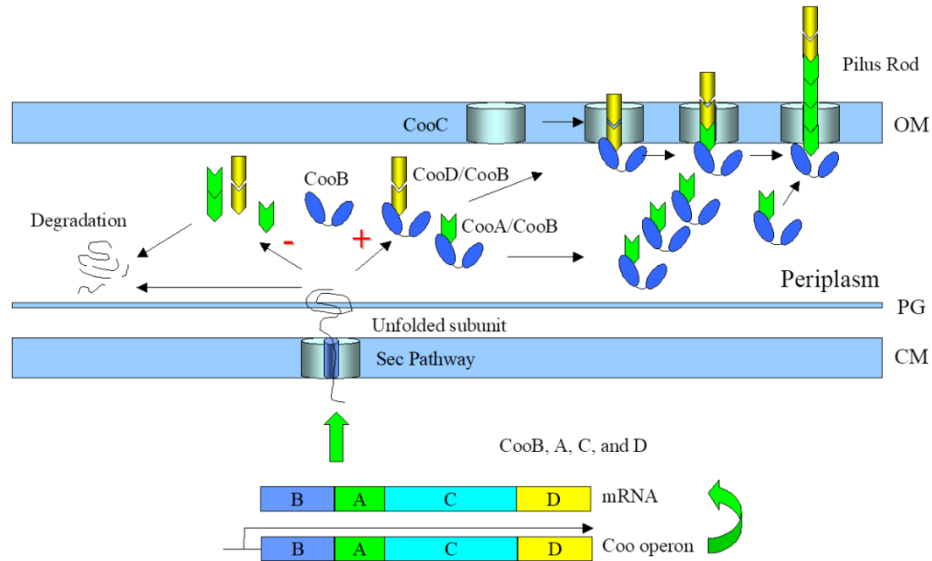


Figure 10. A model of CS1 pilus assembly. Four linked genes, *cooB*, *cooA*, *cooC*, and *cooD*, are arranged in the *coo* operon, encoding the chaperone CooB, the major pilin subunit CooA, the outer membrane usher CooC, and the tip subunit CooD^{24, 47}. All Coo proteins are transported into the periplasm via the Sec Pathway²⁴. Upon entering the periplasm, CooA and CooD form complexes with CooB to prevent degradation⁴⁷. A CooB-CooD complex initiates CS1 pilus assembly by binding to CooC⁶¹. The CooB-CooA complex then binds to CooC, displacing CooD and replacing it with CooA⁵². The repeated addition of CooA at the base of the growing pilus ultimately leads to the extension of the pilus rod across the outer membrane to the cell surface⁵².

2.2 Materials and Methods

2.2.1 Plasmid Construction and Mutagenesis

Mutations in *cooA* and *cooB* were performed using the QuickChange Site-Directed Mutagenesis Kit from Stratagene, and primers (forward and reverse) were obtained from Sigma Genosys. The pQE80CooBA plasmid was used as a template for making the mutations in *cooA* and *cooB*. Trp71 in *CooB* was mutated to Phe (W71F) using the following primers: forward 5'-CCTCTTGCTGTGAATTCGGCACGAGAAGCACCGAGCAC-3' and reverse 5'-GTGCTCCGTGGTGTTCCTCCCAATTCAGAGAAACAGG-3'. Phe84 in *CooA* was mutated to Trp (F84WCooA) using the following primers: forward 5'-CCTGTTTCTGTGAATTGGGCAGGAAAACCACTGAGCAC-3' and reverse 5'-GTGCTCAGTGGTTTTCTGCCCAATTCACAGAAACAGG-3'. Mutant sequences were verified at the Protein Nucleic Acid Laboratory (PNAFL) at Washington University in St. Louis.

2.2.2 Protein Expression and Isolation of W71FCooB–F84WCooA Complex

The DL41 cells containing W71FCooB–F84WCooA complex were streaked out on a Luria-Bertani (LB)/ampicillin (Amp) agar plate and then incubated overnight at 37 °C. The following day, a single isolated colony from the LB/Amp plate was selected and inoculated into 5 mL of LB media supplemented with Amp (100 µg/mL). The culture was then grown at 37 °C, 260 rpm with aeration for 6 to 8 hours. After incubation, 100 mL of LB media (with 100 µg/mL Amp) was inoculated with the starter culture at a 1:100 dilution and grown overnight at 37 °C with aeration.

The expression of the W71FCooB–F84WCooA complex was induced in ECPM1 media supplemented with 100 µg/mL Amp and 1 M MgCl₂/0.45 M CaCl₂. One-hundredth volume of the starter culture was added to 600 mL of ECPM1 and grown at 32 °C with aeration. When the optical

density at 600 nm (OD_{600}) reached 3.0, protein expression was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Gold Biotechnology). The temperature was then reduced to 30 °C, and growth continued for an additional 3 to 4 hours until the OD_{600} doubled.

The cells were harvested by centrifugation using a Beckman Coulter Allegra X-12R Benchtop Centrifuge with a swinging-bucket rotor at 3500 rpm and 4°C for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in buffer 1 (20% sucrose, 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and the solution was stirred for 15 minutes at room temperature. This was followed by centrifugation for 15 minutes at 10,000 rpm and 4 °C using a Beckman Coulter Avanti J-E Centrifuge with a JA-16.250 fixed angle rotor. The cell pellet was then resuspended in buffer 2 (5 mM $MgSO_4$), and the solution was stirred for 15 minutes at 4 °C. Before centrifugation, 0.1 M MES, pH 6.0, was added to achieve a final concentration of 20 mM, and the solution was centrifuged at 10,000 pm and 4 °C for 15 minutes.

2.2.3 Protein Purification of W71FCooB–F84WCooA Complex and W71FCooB

The supernatant was filtered and loaded onto a Hi-Trap SP Sepharose Fast Flow cation exchange column (Cytiva), which was pre-equilibrated with 20 mM MES, pH 6.0 (buffer A) at 4 °C. W71FCooB–F84WCooA complex and W71F CooB were eluted using a NaCl gradient (0-40% NaCl) of buffer B (20 mM MES, pH 6.0, 1 M NaCl) on an AKTA Prime FPLC system. Fractions containing W71FCooB were collected and loaded onto a Superdex-200 16/60 gel filtration column (pre-equilibrated with 20 mM MES, pH 6.0, 0.15 M NaCl) for the final purification step.

W71FCooB–F84WCooA fractions were pooled and dialyzed into 20 mM Tris-Base pH 8.0, with two buffer changes every 12 hours. To remove high molecular weight impurities, the dialyzed

W71FCooB–F84WCooA was purified using a Hi-Trap Q anion exchange column (GE Healthcare), pre-equilibrated with Buffer A (20 mM Tris-Base, pH 8.0), eluted with a NaCl gradient (0-50% NaCl) of buffer B (20 mM Tris-Base, pH 8.0, 1 M NaCl), and then further purified with a Superdex-200 16/60 gel filtration column (pre-equilibrated with 20 mM MES, pH 6.0, 0.15 M NaCl). Protein purity was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentration was determined by the extinction coefficients of $23,505 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (at 280 nm) for W71FCooB–F84WCooA complex and $15,025 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (at 280 nm) for W71FCooB.

2.2.4 Protein Purification of F84WCooA

The W71FCooB–F84WCooA complex was expressed and purified as described in chapter 2.2.2 and chapter 2.2.3. The purified W71FCooB–F84WCooA complex was then incubated overnight in 20 mM MES pH 6.0, with 5 M urea at 4 °C. After dissociation of the complex under denaturing conditions, F84WCooA was purified using a Hi-Trap SP Sepharose Fast Flow cation exchange column (Cytiva) and collected in the flow-through. The concentration of F84WCooA was measured using an extinction coefficient of $8480 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm, and protein purity was determined by SDS-PAGE.

2.2.5 Far UV Circular Dichroism

Circular dichroism measurements were carried out using a Jasco-J-810 spectropolarimeter. Spectra were measured in 20 mM MES, 150 mM NaCl, pH 6.0, using a 0.1 cm pathlength cell at 20 °C with a scan rate of 20 nm per minute and response time of 2 s.

2.2.6 Light Scattering Experiments

Light scattering measurements were conducted using a Cary Eclipse spectrofluorometer, with excitation and emission wavelengths set at 500 nm and slit widths set at 5 nm. All measurements were recorded at 20 °C in a cuvette with a magnetic stir bar. For refolding of F84WCooA without W71FCooB, unfolded F84WCooA in 20 mM MES, 5 M urea, pH 6.0 was diluted 10-fold into a refolding buffer of 20 mM MES, pH 6.0, resulting in final F84WCooA concentrations of 8.80 μ M and 0.88 μ M. For refolding of F84WCooA with W71FCooB, unfolded F84WCooA in 20 mM MES, 5 M urea, pH 6.0, was diluted 11-fold into a refolding buffer of 20 mM MES, pH 6.0 containing 8.80 μ M W71FCooB. The final concentration of F84WCooA was 8.80 μ M.

2.2.7 Tryptophan Fluorescence Experiments

The refolding experiments with F84WCooA were performed by manual mixing, and fluorescence emission spectra were measured using a Cary Eclipse spectrofluorometer with an excitation wavelength set at 295 nm and emission at 320 nm. All measurements were recorded at 20 °C. Fluorescence emission spectra of F84WCooA were measured after a 10-fold dilution of unfolded F84WCooA, which was in 20 mM MES, 5 M urea, pH 6.0 buffer, into a refolding buffer composed of 20 mM MES, pH 6.0, in the presence and absence of W71FCooB. The final concentrations were 1.7 μ M for F84WCooA and 3.7 μ M for W71FCooB. Additionally, fluorescence emission spectra of the purified W71FCooB–F84WCooA complex (1.7 μ M) and W71FCooB alone (1.7 μ M) were also measured.

2.2.8 Kinetic Experiments

The refolding kinetics of F84WCooA were measured using stopped-flow fluorescence spectroscopy, with an excitation wavelength of 295 nm and a cutoff filter of 335 nm. All measurements were conducted at 20 °C. Unfolded F84WCooA in a buffer of 20 mM MES, 5 M urea, pH 6.0, was diluted 10-fold into a refolding buffer of 20 mM MES, pH 6.0, in the absence and presence of increasing concentrations of W71FCooB. The final concentration of F84WCooA was 1.7 μM, and the final concentrations of W71FCooB were 0.9 μM, 1.7 μM, and 3.7 μM. Using OriginLab data analysis software, the data were fitted to the sum of two exponential equations:

$$y = A_1 \times e^{\left(\frac{-x}{t_1}\right)} + A_2 \times e^{\left(\frac{-x}{t_2}\right)} + y_0$$

Where A_1 and A_2 are the amplitudes of the fast and slow phases, x is the time, t_1 and t_2 are the time constants of the fast and slow phases, and y_0 is the offset.

2.3 Results

To determine if the CS1 pilus chaperone CooB facilitated the refolding of the CS1 pilus subunit CooA, we endeavored to monitor only changes in tryptophan fluorescence of CooA during its folding in the presence of CooB. Previous studies on the refolding of pilus subunits PapE and FimG were conducted by monitoring the tryptophan fluorescence of the subunits during the folding process, both in the absence and presence of a chaperone that lacks tryptophan.

Since CooA lacks tryptophan, we mutated Phe-84 to tryptophan (F84W) because the corresponding position in CfaB (CFA/I pilus subunit), a homolog of CooA, contains a tryptophan residue. Wild-type CooB contains one tryptophan residue (Trp-71). Therefore, we generated a CooB mutant in which Trp-71 was mutated to phenylalanine (W71F) to eliminate the fluorescence contribution from CooB, allowing us to monitor solely the tryptophan fluorescence in F84WCooA

even in the presence of CooB. We verified that the mutations did not cause significant structural changes, as demonstrated by the similar circular dichroism (CD) spectra of the W71FCooB–F84WCooA and W71FCooB mutants compared to the wild-type CooBA and CooB (Figure 11(A) and (B)).

The W71FCooB–F84WCooA complex and W71FCooB were purified as described in Chapter 2.2.3. To isolate F84WCooA from the W71FCooB–F84WCooA complex, we denatured the complex in 5 M urea and separated F84WCooA from W71FCooB using an SP cation exchanger column. Figure 12(A) and (B) present the SDS-PAGE analysis of the purified W71FCooB–F84WCooA complex, W71FCooB, and F84WCooA. Two distinct bands are observed on the SDS-PAGE: one at a molecular weight of 25 kDa, corresponding to W71FCooB, and the other at 15.2 kDa, corresponding to F84WCooA.

F84WCooA is unfolded at 5 M urea, as indicated by no changes in the emission spectra (emission maxima = 360 nm) up to 8 M urea. F84WCooA was then refolded by a 1:10 urea dilution from 5 M to 0.5 M. At an F84WCooA concentration of 8.8 μ M, we observed a time-dependent increase in light scattering, indicating aggregation of the subunit during refolding (Figure 13 (A)). This aggregation was concentration-dependent, as refolding at a ten-fold lower concentration (0.88 μ M) largely eliminated the aggregation phenomenon. In the presence of an equivalent amount of CooB (8.8 μ M), the light scattering was significantly reduced, suggesting that W71FCooB functions as a classical molecular chaperone by binding to unfolded F84WCooA and preventing the aggregation process.

Figure 13(B) illustrates the tryptophan fluorescence emission spectra during the refolding of F84WCooA, showing significant changes in fluorescence as F84W CooA was diluted from a high urea concentration (5 M) to a low urea concentration (0.5 M). The emission maximum shifted

from 360 nm at 5 M urea to 340 nm at 0.5 M urea. In the presence of W71FCooB, the emission maximum shifted even further to 320 nm, accompanied by an increase in fluorescence intensity. The emission maxima of the refolded F84WCooA with W71FCooB were similar to that of the purified native W71FCooB–F84WCooA complex, suggesting that the tryptophan achieves a similar final folded environment. This indicates that W71FCooB reduces aggregation, allowing F84WCooA to achieve a more native-like structure.

To investigate whether the chaperone W71FCooB catalyzes the refolding of F84WCooA, we used stopped-flow tryptophan fluorescence to monitor the refolding kinetics of F84WCooA alone and in the presence of W71FCooB. The refolding kinetics, both with and without W71FCooB, were fitted to a sum of two exponentials, indicating two observable phases during the refolding process: a fast phase and a slow phase (Figure 14). We observed no increase in the rate constants of the two kinetics phases for F84WCooA refolding in the presence of W71FCooB (Table 3), suggesting that the rate of refolding of F84WCooA did not accelerate with W71FCooB. However, despite the unchanged rate constants, we observed a ten-fold increase in the total amplitude of the fluorescence from 0.026 ± 0.0012 (at 0 μM W71FCooB) to 0.28 ± 0.0023 (at 3.7 μM W71FCooB). This increase in amplitude is likely due to a reduction in aggregation, leading to a higher yield of folded F84WCooA, consistent with the results from light scattering experiments (Figure 13 (A))

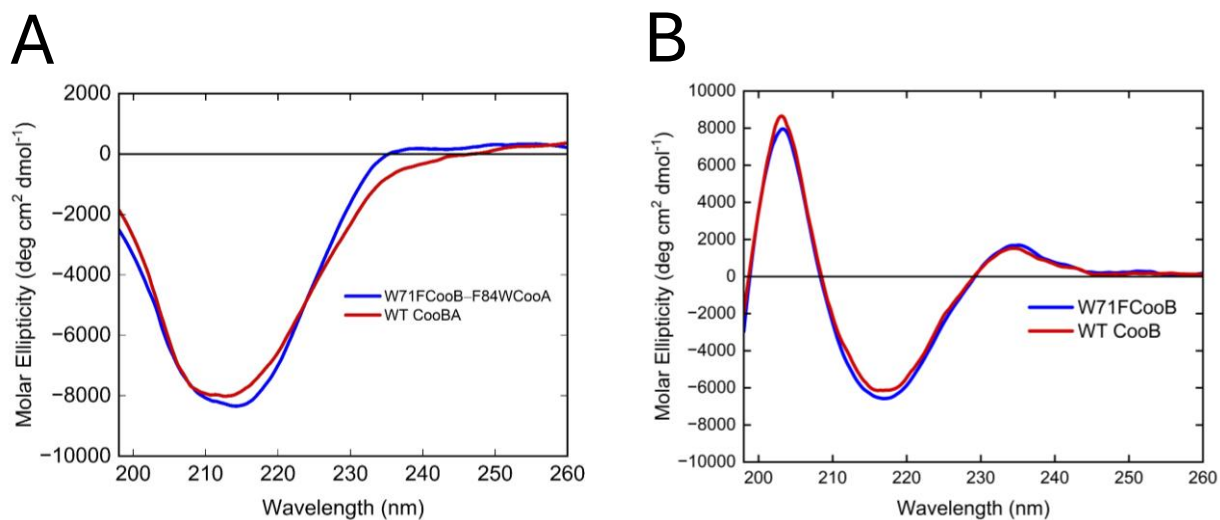


Figure 11. (A) Circular dichroism spectra of W71FCooB-F84WCooA (blue) and wild-type CooBA (red) (B) Circular dichroism spectra of W71FCooB (blue) and wild-type CooB (red). Spectra were recorded at 20 °C in 20 mM MES, 150 mM NaCl, pH 6.0.

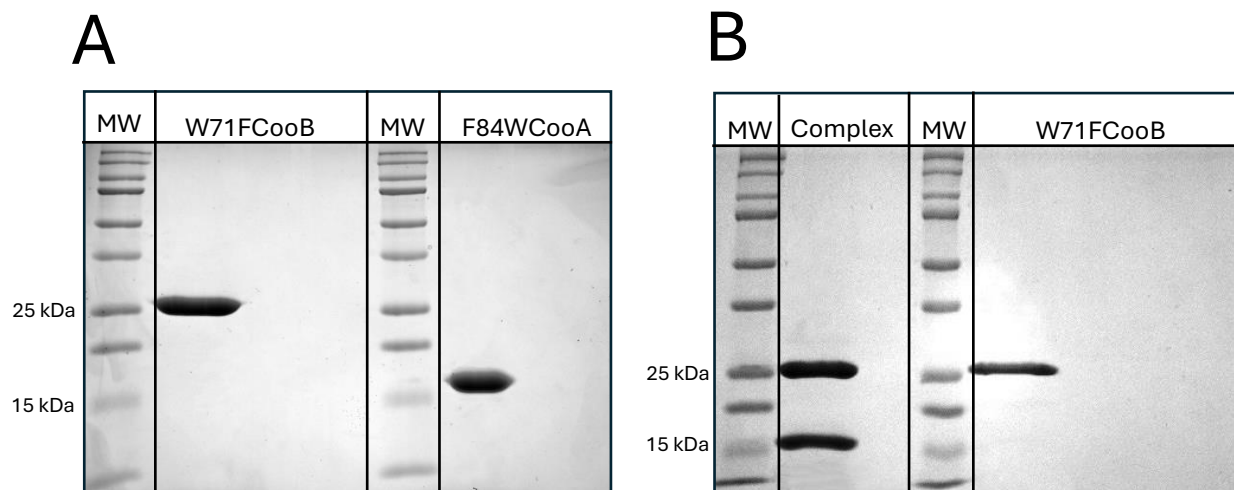


Figure 12. (A) SDS-PAGE analysis of purified W71FCooB (25 kDa) and F84WCooA (15.2 kDa) (B) SDS-PAGE analysis of purified W71FCooB-F84WCooA complex. The gel shows two distinct bands at 25 kDa and 15.2 kDa, corresponding to W71FCooB and F84WCooA.

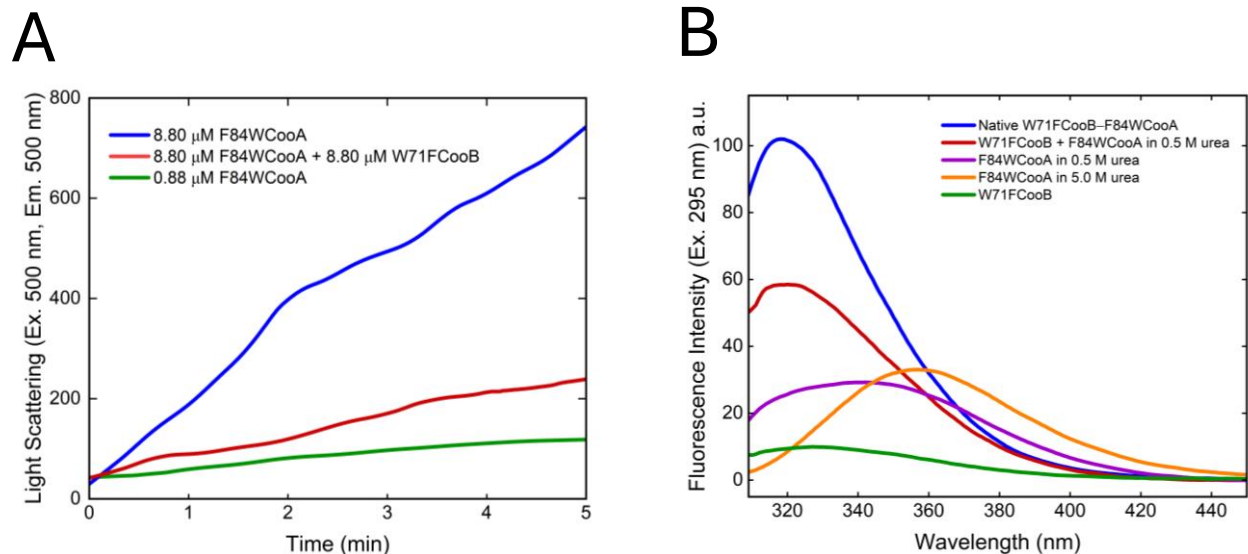


Figure 13. (A) Light scattering of F84WCooA after 10-fold dilution of urea from 5 M to 0.5 M, with a final F84WCooA concentration of 8.80 μM in the absence (blue) and presence (red) of 8.80 μM W71FCooB, and a final F84WCooA concentration of 0.88 μM (green) in the absence of W71FCooB. Light scattering was measured at 20 $^{\circ}\text{C}$ using an excitation and emission wavelength of 500 nm. (B) Tryptophan fluorescence emission spectra of F84WCooA (1.7 μM) in 5 M urea (orange), in 0.5 M urea (purple), and the presence of 3.7 μM W71FCooB (red). The fluorescence emission spectra of the purified native W71FCooB-F84WCooA complex (1.7 μM) and W71FCooB (3.7 μM) were shown in blue and green, respectively. Spectra were recorded using an excitation wavelength of 295 nm, and all experiments were performed in 20 mM MES, pH 6.0, at 20 $^{\circ}\text{C}$.

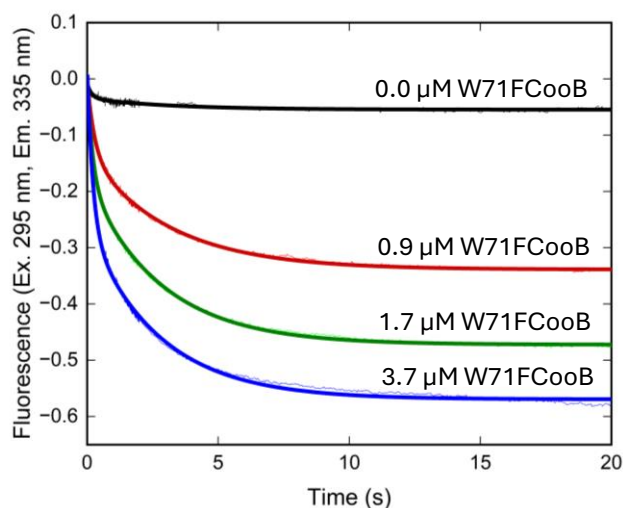


Figure 14. Kinetics of refolding of F84WCooA after a 1:10 dilution (final F84WCooA concentration of 1.7 μM) from 5 M to 0.5 M urea in the absence of W71FCooB (black) and the presence of 0.9 μM (red), 1.7 μM (green), 3.7 μM (blue) W71FCooB. The data were fit to a sum of two exponentials, and the fits are the solid lines through the data.

Table 3.

Rate Constants and Amplitudes of F84WCooA Refolding

CooB (μM)	A_1	k_1 (s^{-1})	A_2	k_2 (s^{-1})
0.0	0.026 ± 0.0012	4.05 ± 0.23	0.020 ± 0.0096	0.32 ± 0.021
0.9	0.13 ± 0.0012	4.00 ± 0.081	0.21 ± 0.00091	0.32 ± 0.0029
1.7	0.18 ± 0.0013	4.71 ± 0.069	0.29 ± 0.00088	0.36 ± 0.0022
3.7	0.28 ± 0.0023	4.62 ± 0.041	0.30 ± 0.0016	0.37 ± 0.0032

* A indicates amplitude, and k indicates the rate constant.

2.4 Discussion

Ensuring that proteins fold into their native three-dimensional conformation is crucial. Misfolded proteins can aggregate, and the accumulation of these aggregates can lead to serious diseases³⁴. However, two primary mechanisms facilitate correct protein folding and prevent aggregation. The first mechanism involves molecular chaperones, which are proteins that bind to nascent or partially folded polypeptides, preventing nonspecific aggregation and promoting proper folding³⁵. The second mechanism involves protein folding catalysts, which accelerate the rate-limiting steps in protein folding, such as disulfide bond formation and proline *cis-trans* isomerization³⁵. These catalysts not only enhance the yield of correctly folded proteins but also increase the overall rate of the folding process³⁵.

Classical molecular chaperones increase the amount of correctly folded proteins but do not accelerate the folding process³⁵. However, recent studies suggest that pilus chaperones represent a new type of chaperone that not only prevents aggregation, like classical molecular chaperones,

but also functions as a protein folding catalyst to accelerate the folding rate ^{44, 62}. Type 1 pilus chaperone FimC and P pilus chaperone PapD are well-studied examples of this new class of pilus chaperones. They have been shown to catalyze the folding of pilus subunits by the chaperone-usher pathway.

Pilus chaperones of the alternate chaperone-usher pathway exhibit low sequence homology to those in the chaperone-usher pathway. No studies have demonstrated that pilus chaperones from the alternate chaperone-usher pathway catalyze the folding of the pilus subunit. The CS1 pilus system is one of the best characterized within the alternate chaperone-usher pathway and can serve as a model system for studying pilus subunit folding. Therefore, my thesis investigates whether the chaperone in the CS1 pilus system functions as a protein folding catalyst to accelerate the folding rate of the CS1 pilus subunit.

We unfolded the CS1 pilus subunit F84WCooA in a high concentration of urea denaturant and subsequently refolded the subunit by diluting it to a low urea concentration. During the refolding process, we observed an increase in light scattering, indicating subunit aggregation. However, when W71FCooB was present, there was a decrease in light scattering, suggesting a reduction in aggregation. Additionally, we monitored the refolding kinetics of F84WCooA in the presence and absence of the CS1 chaperone W71FCooB at a low urea concentration using stopped-flow tryptophan fluorescence. We observed a significant increase in the amount of folded F84WCooA in the presence of W71FCooB, as determined by the increased amplitude of fluorescence change, but no increase in the refolding rate was observed.

The light scattering and refolding kinetic data presented here indicate that the CS1 pilus chaperone W71FCooB facilitates subunit folding similarly to classical molecular chaperones by preventing subunit aggregation rather than acting as a protein folding catalyst. This function

distinguishes it from known pilus chaperones like FimC (for type 1 pilus) and PapD (for P pilus), which have been shown to catalyze the folding of subunits^{44, 62}.

The formation of a disulfide bond between two cysteine residues in pilus subunits is a slow, rate-limiting step in protein folding, requiring a protein folding catalyst to accelerate the reaction⁶². However, whether the disulfide bond formation in the pilus subunit occurs before chaperone binding or after the pilus subunit folds on the chaperone template is unclear. A study on the refolding of the type 1 pilus subunit FimA suggested that the disulfide oxidoreductase DsbA first catalyzes the oxidation of cysteine residues in the pilus subunit to form disulfide bonds, which subsequently bind to the chaperone FimC, thereby catalyzing the refolding of FimA⁶³.

When choosing a model system to study subunit folding, the CS1 pilus system is unique in that it is simple (requiring only four genes for pilus assembly⁴⁷), but importantly, the pilus subunits do not have cysteine residues, which precludes the need for DsbA to assist in the folding process. Thus, we expected that the chaperone would, similar to PapD and FimC, catalyze the refolding process without the necessity to form a disulfide bond. Our data suggests that once the subunit is imported into the periplasm, it can achieve largely correct topology, although it is missing the seventh G β -strand. However, aggregation occurs without the donation of the G β -strand from the chaperone to complete the subunit's immunoglobulin-like (Ig-like) folding. Upon import into the periplasm, F84WCooA folding is immediately followed by binding to the chaperone, preventing off-pathway aggregation. In addition, key residues (Arg-8 and Lys-112) crucial for pilus folding in the classical chaperone-usher pathway chaperones, such as PapD, are absent in the alternate chaperone-usher pathway chaperones. Thus, we believe that the alternate chaperone-usher pathway is an example of convergent evolution—achieving the same goal (pilus formation) through similar but distinct mechanisms.

2.5 Conclusion

Molecular chaperones play a crucial role in protein folding by preventing off-pathway reactions that lead to aggregation³⁵. In pilus biogenesis, pilus chaperones are thought to facilitate pilus subunit folding by preventing subunit aggregation during the folding process⁴⁴. Previous studies on the folding of the type 1 pilus subunit and P pilus subunit, which are well-studied pili assembled by the classical chaperone-usher pathway, have shown that pilus chaperones represent a new type of protein-folding chaperone^{44,62}. These chaperones act as the protein-folding catalyst that prevents subunit aggregation while catalyzing the folding of the subunit^{44,62}. In this study, we aimed to determine if the pilus chaperones from the alternate chaperone-usher pathway catalyze subunit folding. The CS1 pilus, one of the best-characterized pili assembled by the alternate chaperone-usher pathway, serves as a model system for studying pilus subunit folding. Our findings indicate that refolding the CS1 subunit CooA from denaturing conditions in the presence of chaperone CooB reduces the aggregation and increases the amount of folded CooA, but does not increase the refolding rate. These results suggest that CS1 chaperone CooB is not a protein-folding catalyst. Instead, it acts as a classical molecular chaperone by donating its own G β -strand to complete the subunit Ig-like fold, thereby preventing aggregation and promoting the formation of the folded protein. Although our findings indicate that CooB, the chaperone of the alternate chaperone-usher pathway, does not catalyze pilus subunit folding, no studies have elucidated how pilus chaperones facilitate the folding of pili in this pathway. Therefore, future research could focus on other pilus systems within the alternate chaperone-usher pathway, such as CFA/I and CS2 pili, to fully understand the subunit folding mechanisms and the role of chaperones in the folding process.

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