Mechanism of FimI, the assembly termination subunit of the type 1 pili from uropathogenic *Escherichia coli*

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1. SUMMARY

The most frequently diagnosed kidney and urologic diseases in humans are urinary tract infections (UTIs). UTIs afflict millions of people every year, particularly women and aged people. More than 80% of all UTIs are caused by uropathogenic strains of *Escherichia coli* bearing adhesive filaments termed type 1 pili, which mediate bacterial attachment to the host cell surface. Type 1 pili are filamentous, supramolecular protein complexes composed of the four structural subunits FimH, FimG, FimF, and FimA. Together with the periplasmic chaperone FimC and the assembly platform ("usher") FimD, the structural subunits are encoded by the *fim* gene cluster. The structural subunits are secretory proteins whose folding in the periplasm is catalysed by the dithiol oxidase DsbA and the pilus assembly chaperone FimC. The subunits are structurally homologous proteins sharing an incomplete immunoglobulin-like (Ig-like) fold in which the C-terminal β-strand G is missing. The folded subunits form heterodimeric complexes with FimC in the periplasm, in which FimC donates an extended polypeptide segment to the bound subunit and thereby completes the subunit’s fold, a mechanism termed “donor strand complementation” (DSC). The assembly platform FimD in the outer membrane then catalyses pilus assembly by binding individual FimC-subunit complexes and enabling the incorporation of the subunit into the growing pilus. In this non-covalent polymerization process termed “donor strand exchange” (DSE), an N-terminal extension (donor strand) in the next, FimC-bound subunit to be incorporated displaces the FimC chaperone capping the last subunit at the growing end of the pilus and inserts as a β-strand into this subunit to complete its fold. The ability of the incoming subunit to displace FimC from the growing pilus end depends on the accessibility of a specific binding site in the last pilus subunit termed P5. It had been postulated that DSE is initiated at P5 and proceeds via a zip-in-zip-out mechanism eventually displacing FimC at the growing pilus end.

The aim of the present work was the structural and functional characterization of the pilus subunit FimI, a protein that had never been identified as a component of type 1 pili but is encoded in an operon together with the main structural pilus subunit FimA on the *fim* gene cluster. Initial experiments on FimD-catalyzed pilus assembly *in vitro* in our laboratory had
indicated that FimI terminates pilus assembly and contributes to a stable anchoring of the pilus to the outer membrane. Specifically, the data predicted that the \textit{in vivo} concentrations of FimI are much lower than those of the main subunit FimA, and that FimI stops pilus assembly in a stochastic chain termination reaction. In the present thesis, we tested the hypothesis that slow or inefficient folding of FimI compared to FimA is responsible for its low \textit{in vivo} concentration. We could show that FimI interacts with the periplasmic folding catalysts DsbA and FimC in a similar manner as the main subunit FimA, and that DsbA-catalyzed disulfide bond formation is required for FimC-catalyzed folding of FimI. Together with the known \textit{in vivo} concentrations of DsbA and FimC, the obtained rates of DsbA-dependent oxidation and FimC-catalyzed folding of FimI predicted that the \textit{in vivo} half-life of FimI folding is even shorter than folding of FimA (0.7 s compared to 1.1 s, respectively). Specifically, FimI was oxidized by DsbA about 30 times faster than FimA, while FimC-catalyzed folding of FimI and FimA proceeded equally fast. Together, the results exclude inefficient \textit{in vivo} folding of FimI as the cause of its low \textit{in vivo} concentrations, suggesting that FimI may be less efficiently translated or more efficiently degraded \textit{in vivo} relative to FimA.

FimI is unique in having the longest donor strand among the other pilus subunits. A N-terminally truncated variant of FimI depleted of its donor strand, termed FimI\(_t\), was characterized. While oxidation of FimI\(_t\) by DsbA was 3 times slower than oxidation of FimI, FimC-catalyzed folding of FimI\(_t\) was 10-fold faster than folding of full-length FimI. Moreover, the X-ray structure of the FimC-FimI\(_t\) complex at 1.75 Å resolution, and the structure of FimC-FimI\(_t\) bound to FimD\(_{NTD}\), the N-terminal substrate recognition domain of FimD, at 1.70 Å resolution were solved in collaboration with Dr. M. Schärer and Dr. G. Capitani (Paul Scherrer Institute). The structures revealed the specific interactions between binding FimI\(_t\) and FimC, in particular the mode of FimC donor strand insertion into the pilin domain of FimI parallel to the C-terminal F-strand of FimI\(_t\). The structural analysis of the ternary FimC-FimI\(_t\)-FimD\(_{NTD}\) complex showed the residues Phe4, Phe8, Phe22 from the N-terminal FimD\(_{NTD}\) tail form a hydrophobic cluster with the FimC residues Leu32 and Ile90 that had previously also been observed in the ternary complexes with other type 1 pilus subunits. However, the structure of the FimC-FimI\(_t\)-FimD\(_{NTD}\) complex revealed a closed P5 pocket in FimI\(_t\), while the
P5 pocket stayed open in the FimC-FimI complex and all previously characterized ternary complexes between FimC, FimD_{NTD} and other subunits. The results indicate that FimD_{NTD} specifically induces a conformational transition in FimC-bound FimI that closes the P5 pocket of FimI. This mechanism prevents the displacement of FimC from FimI by DSE and explains why pilus assembly can no longer proceed once FimI is incorporated.
2. ZUSAMMENFASSUNG

dann FimC von wachsenden Pilusende in einem Mechanismus verdrängt, der analog zu einem sich an einem Ende schliessenden und am andern Ende öffnenden Reisverschluss ist.


FimI ist einzigartig, weil es den längsten Donorstrang im Vergleich zu den anderen Pilusuntereinheiten hat. Es wurde eine N-terminal verkürzte Variante von FimI mit fehlendem Donorstrang charakterisiert, die als FimI₄ bezeichnet wurde. Während die
Zusammenfassung

3. INTRODUCTION

3.1. Type 1 pili from uropathogenic *E.coli*

The most frequently diagnosed kidney and urologic disease in humans is urinary tract infection (UTI) and by far its most common etiologic agent is *Escherichia coli* (Kahlmeter and Eco.Sens 2003, Ronald 2003). UTIs are classified according to the site of infection: cystitis (the bladder), pyelonephritis (the kidney) and bacteriuria (the urine) (Foxman 2003). They are considered to be the most common infections in human, which afflict millions of people, particularly women. Important virulence factors of gram-negative uropathogenic *E.coli* are type 1 pili encoded by the *fim* gene cluster (Connell et al. 1996). They enable the bacterium cells to attach to the urinary epithelial cells of the host and play thus an important role in the establishment of the infections and colonization. The binding to mannosylated receptors present on epithelial bladder host cells is mediated through the FimH adhesin located at the very tip of the pilus. Type 1 pili are anchored within the outer membrane of the bacterium, reach into the extracellular space, and have a rod-like appearance with a pilus length of up to 2 µm and a diameter of 7 nm (Figure 1A) and they can be removed from the cell surface by mechanical shearing (Hahn et al. 2002) (Figure 1B).

![Figure 1A: Electron micrographs of a single *E. coli* cell and isolated type 1 pili.](image1a)

![Figure 1B:](image1b)
microscopy (TEM) (Hahn et al. 2002). The upper figure in a rectangle shows detailed view of rigid, rod-like type 1 pili spread on the whole *E.coli* cell surface. 1B: TEM image of isolated type 1 pili negatively stained with uranyl formate. The isolated pili fragments are indicated by the white arrow. The scale bars represent: (1A) 0.7 μm; (1B) 0.12 μm. Figures were taken from (Hahn et al. 2002).

Type 1 pili from uropathogenic *Escherichia coli* (UPEC) strains are large, hetero-oligomeric protein filaments, which are assembled by the chaperone-usher pathway (CUP). They consist of 5 structural subunits: FimA, FimF, FimG, FimH and FimI, which are together with chaperone-usher pair encoded by the *fim* gene cluster. Type 1 pili are composed of up to 3000 subunits of the main structural subunit FimA, which forms the pilus rod (Brinton 1965, Jones et al. 1995, Hahn et al. 2002) (Figure 2A). Two linker subunits FimF, FimG and the adhesin FimH form a short flexible fibrillar tip in which FimF and FimG connect the pilus rod to a single copy of FimH (Jones et al. 1995, Hahn et al. 2002, Le Trong et al. 2010). FimH consists of two domains: a pilin domain (FimHₚ) similar to all other pilus subunits and a receptor-binding domain referred to as the lectin domain (FimHₗ) (Choudhury et al. 1999). FimHₗ mediates binding to D-mannose oligosaccharides (Abraham et al. 1988, Krogfelt et al. 1990) and thereby enable the bacteria to colonize various host tissues (Brinton 1965, Ofek et al. 1977, Duguid 1980).

Another one of the best characterized fimbrial adhesin is chromosomally encoded pyelonephritis-associated pilus (Pap or P fimbriae). In uropathogenic *Escherichia coli*, P pili mediate pyelonephritis by binding to the globoseries of glycolipids in the human kidney (Lund et al. 1985, Kuehn et al. 1992, Roberts et al. 1994, Foxman 2002). P pilus consists of 5 Ig-like pilin subunits (PapF, PapE, PapK, PapA, PapH) and the adhesin called PapG (Figure 2B). The right-handed helical rod is made up of thousands of copies of main structural subunit PapA (Baga et al. 1984, Gong and Makowski 1992, Bullitt et al. 1996, Verger et al. 2007). The helical tip fibrillum is formed by PapG (Dodson et al. 2001), PapF, multiple PapEs, and PapK (Hultgren et al. 1991, Sauer et al. 1999). The minor subunit PapK links the tip of the fibrillum to the rod, while PapF serves as an adaptor between the tip fibrillum and the adhesin PapG (Lindberg et al. 1987, Jacob-Dubuisson et al. 1993). The P pilus growth is terminated by a single copy of PapH subunit (Baga et al. 1987, Verger et al. 2006), whose homologue in type 1 pili is FimI subunit. Upon the subunits transfer to the periplasm, they are assembled through the actions of the periplasmic chaperone (PapD) and the integral outer membrane
 usher (PapC). Type 1 and P pili are prototypes for understanding assembly by the chaperone-usher pathway.

Figure 2: Schematic representation of type 1 and P pilus assembly according to the chaperone-usher pathway. 2A: Type 1 pilus tip fibrillum subunits adhesin FimH (green), FimG (orange), FimF (brown) and pilus rod subunit FimA (cyan) enter the periplasm in an unfolded conformation, where they bind the chaperone FimC (yellow) in 1:1 complexes after they are oxidized by DsbA. Formation of chaperone-subunit complex is mediated through the donor-strand complementation mechanism. After FimC-catalyzed folding, the chaperone-subunit complexes diffuse to the outer membrane usher, FimD. During pilus assembly the donor strand of FimC is replaced by the N-terminal extension of the following subunit in a mechanism called donor-strand exchange. FimI (grey) is the last subunit, which is incorporated into the pilus and terminates the pilus assembly (Ignatov 2009). 2B: P pilus is composed of the pilus rod subunit PapA (cyan), tip fibrillum subunits: PapG (green), PapF (orange), PapE (brown), PapK (violet) and the termination subunit PapH (grey). Chaperone PapD and usher PapC are shown in yellow and blue, respectively. The figures were created according to (Nishiyama et al. 2005, Geibel et al. 2013).
The pilus subunits, called pilins, are synthesized in the cytoplasm and exported to the periplasm via the general secretory pathway through the inner membrane (IM) in an unfolded, reduced conformation (Figure 2A). All pilus subunit precursors contain an N-terminal signal sequence, which directs the newly synthesized subunits to the translocon SecYEG. In the periplasm, the invariant, structural disulfide bond in each pilus subunit is formed by oxidation through the periplasmic dithiol oxidase DsbA. The formation of a single disulfide bond is conserved in all structural subunits. The unfolded disulfide-intact subunits are recognized by the periplasmic pilus chaperone FimC, which catalyzes subunit folding up to $10^4$-fold and forms 1:1 chaperone-subunit complexes (Vetsch et al. 2004, Erilov 2007, Crespo et al. 2012). In the periplasm, folding and stability of the subunits require formation of a binary complex with the FimC chaperone (Barnhart et al. 2000, Vetsch et al. 2004).

3.2. Parallel and anti-parallel donor strand complementation

The crystal structure of the FimC-FimH complex revealed that the pilin domain of FimH adopts a truncated, immunoglobulin-like (Ig-like) fold, in which the C-terminal G strand of the subunit is missing (Choudhury et al. 1999, Sauer et al. 1999). The same pilin fold was later confirmed for all other subunits by X-ray crystallography (Erilov 2007, Eidam et al. 2008, Ignatov 2009, Crespo et al. 2012). The thermodynamic stability of assembled pilus subunits is higher in contrast to stability of free pilus subunits (Vetsch et al. 2002, Vetsch et al. 2004, Erilov 2007). This explains the high tendency of structural pilus subunits to aggregate non-specifically in the absence of stoichiometric amounts of FimC (Vetsch et al. 2002). As first shown by studies on the Pap and Fim systems, in the complex with the pilus subunit, G β-strand of the N-terminal domain of the chaperone inserts a conserved motif of 4 alternating hydrophobic residues (called “P1 to P4 residues”) into 4 binding pockets in the hydrophobic groove of the pilus subunits (termed “P1 to P4 binding pockets”). The donated G β-strand of FimC makes hydrogen bonding interactions with the F β-strand of subunit and, thus completing subunit’s Ig fold. The donated G β-strand of FimC fold was termed “donor strand” and thus the mechanism of completing pilin Ig fold by FimC donor strand was termed “donor-strand complementation” (DSC) (Choudhury et al. 1999, Sauer et al. 1999).
The inserted donor strand of FimC, between the first (A β-strand) and last (F β-strand) of subunit, runs in parallel orientation compared to the F β-strand of the subunit’s fold leading to a non-canonical Ig-like fold (Figure 3A) (Choudhury et al. 1999, Sauer et al. 1999, Barnhart et al. 2000). After DSC, the chaperone-subunit complex is bound by the assembly platform FimD in the outer membrane which is termed “usher”. The incoming chaperone-subunit complexes bind to the N-terminal periplasmic domain of FimD (FimD NTD) and are incorporated into the growing pilus via a noncovalent polymerization process called “donor-strand exchange” (DSE) (Soto et al. 1998, Sauer et al. 2002, Zavialov et al. 2003, Vetsch et al. 2006). All pilus subunit, except FimH, have an unstructured N-terminal extension of 15-20 amino acid residues, which is not part of the Ig-like fold (Choudhury et al. 1999, Puorger et al. 2008). As shown in FimC donor strand sequence, these extensions also possess the pattern of alternating hydrophobic residues (named “P1 to P5 residues”) and are termed “donor strand” (ds). In the assembled pilus, FimC donor strand is replaced by ds of subunit, which is next in pilus assembly. Interestingly after DSE, the ds now runs antiparallel to F β-strand of the subunit’s fold (Figure 3B). It was shown that antiparallel donor strand complementation is a consequence of the high stability of the assembled type 1 pilus subunits against dissociation (Puorger et al. 2008). As recently revealed, a bimolecular complex between truncated variant of FimG without ds (FimGt) and the 15-residue donor strand peptide of FimF (dsF) subunit, FimGt-dsF complex, is the kinetically and thermodynamically most stable non-covalent protein-ligand complex known up to date (Puorger et al. 2008, Giese et al. 2012).
Figure 3: Parallel and anti-parallel donor strand complementation. 

3A: The crystal structure of the FimC-FimI-FimA<sub>t</sub> complex at 2.8 Å resolution shows parallel donor strand complementation of chaperone-subunit complex (Ignatov 2009). FimI subunit is shown in grey, FimC in pale-yellow and FimA<sub>t</sub> in cyan. The donor strand of FimC, residues 100-117, is indicated by red arrow. 

3B: The crystal structure of the FimF-FimF-FimC complex extracted from FimH-FimG-FimF-FimF-FimC complex at 2.7 Å resolution (PDB identifier 3JWN) shows antiparallel donor strand complementation of subunit-subunit complexes (Le Trong et al. 2010). The donor strands of FimF subunits, residues 1-12, are shown by red arrows in
antiparallel orientation to the sixth β-strand of the accepting subunit (FimF). FimF subunits are depicted in brown, FimC in pale-yellow. Figures were created according to (Le Trong et al. 2010) and (Ignatov 2009).

3.3. A current model for subunit incorporation cycle

FimD catalyzes pilus assembly independent of cellular energy sources and enables the translocation of the growing fibre across the outer membrane in a self-energized process (Jacob-Dubuisson et al. 1994, Nishiyama et al. 2008). The usher consists of 5 functional domains: an N-terminal periplasmic domain (FimD<sub>NTD</sub> or NTD), two carboxy-terminal periplasmic domains (CTD1 and CTD2), a β-barrel pore domain that, in the resting state, is occluded by a plug domain (Phan et al. 2011). While FimD<sub>NTD</sub> is responsible for binding chaperone-subunit complexes (Nishiyama et al. 2003, Ng et al. 2004, Nishiyama et al. 2005), the C-terminal domains of FimD have poorly understood functions (So and Thanassi 2006, Ford et al. 2010). The transmembrane 24-stranded β-barrel domain forms a channel through which the folded and assembled pilus subunits are translocated to the extracellular space (Remaut et al. 2008, Huang et al. 2009). The region between strands β6 and β7 of the β-barrel holds a 80-residue plug domain. The FimC–FimH complex is the first chaperone–subunit complex recruited to bind to the FimD<sub>NTD</sub> and necessary to transfer FimD into an active catalyst (Nishiyama et al. 2008). The FimH lectin domain is essential for the activation of FimD, therefore the ability to convert FimD into a high-efficiency assembly catalyst is restricted to FimC–FimH (Nishiyama et al. 2008). The structure of activated FimD has been solved and shows that FimH<sub>L</sub> is inside the barrel and has displaced the usher plug domain from barrel lumen to the periplasm (Phan et al. 2011). However how the plug domain relocates to a proximal position to the FimD<sub>NTD</sub> is unknown. A current model for subunit-incorporation cycle (Figure 4B) was formulated based on the crystal structure of the elongation complex FimC–FimD–FimF–FimG–FimH (Figure 4A) showing a post-initiation state during pilus biogenesis (Geibel et al. 2013). The first step in pilus assembly is binding incoming chaperone–pilin subunits to the usher FimD<sub>NTD</sub> and then transfer after DSE to the usher CTDs during assembly. This transfer requires a rotation of the FimD<sub>NTD</sub> -bound chaperone–subunit complex by about 100°–120° after DSE (Geibel et al. 2013). These
conformational changes are important to prevent backsliding of the nascent pilus through the FimD pore (Geibel et al. 2013). FimC has no binding affinity for the usher CTDs on its own, and so dissociates from the complex (Geibel et al. 2013).

4A

Figure 4: The crystal structure of FimC–FimD–FimF–FimG–FimH and a model for subunit incorporation cycle. 4A: The structure of FimC-FimD-FimF-FimG-FimH shows an elongated complex of an adhesin subunit FimH (green), FimG (orange), FimF subunit (brown) in a complex with FimC (pale-yellow) bound to C-terminal domains of FimD. Color-coding of FimD domains: pale green, magenta, blue, cyan and violet for NTD, Plug, FimD pore, CTD1 and CTD2 domain, respectively. Symbols E, OM and P are used for extracellular space, outer membrane and periplasm, respectively. 4B: Schematic diagram of proposed subunit incorporation cycle. Color scheme is as in Figure 4A. The groove in all subunits is highlighted by
black. The first step is the recruitment of FimC-FimG complex to the FimD<sub>NTD</sub>. This positions FimG and FimH for donor-strand exchange (DSE) and causes that the chaperone FimC is released from FimH-FimC complex (steps 2 and 3). Finally, in step 4, transfer of the nascent pilus from the NTD to the CTDs occurs concomitantly to translocation of the nascent pilus through the pore. Figures were modified according to (Geibel et al. 2013).

### 3.4. Genetic organization of the <i>fim</i> gene cluster and <i>pap</i> operon

The <i>fim</i> gene cluster encodes 9 genes necessary for the synthesis, assembly, and regulation of the type 1 pilus (Figure 5A). The genes <i>fimB</i> and <i>fimE</i> are encoding for the regulatory proteins FimB and FimE, which have 48% amino acid homology with each other (Klemm 1986). Then there is a 314 bp invertible DNA fragment called <i>fimS</i>. The genes <i>fimB</i> and <i>fimE</i> lie upstream of <i>fimS</i> in the “ON” orientation and are transcribed from separated promoters (P<sub>B</sub> and P<sub>E</sub>) (Blomfield 2001). The <i>fimS</i> invertible element lies in the <i>fimE-A</i> intergenic region and contains the promoter (P<sub>A</sub>) for expression of genes <i>fimA</i>, <i>fimI</i> and <i>fimC</i> with 9 bp inverted repeats (IRs). The FimB protein binds equally well <i>in vitro</i> and <i>in vivo</i> to its target sites located at the functionally equivalent left (IRL) and right (IRR) inverted repeats that flank the switch (Dove and Dorman 1994, Bollinger et al. 2006). The <i>fimA</i> promoter sequence undergoes site-specific recombination, positioning the invertible element in either the phase “ON” or phase “OFF” orientation (Blomfield 2001, Schwan 2011). In phase “ON” cells, a promoter within the invertible element directs the transcription of the <i>fim</i> structural genes <i>fimA</i>, <i>fimI</i> and <i>fimC</i>, whereas in phase “OFF” cells transcription of these fimbrial genes is silenced (Blomfield 2001). This switching phenomenon is known as phase variation. FimB and FimE are members of the tyrosine recombinase family (Esposito and Scocca 1997). Only the FimB recombinase efficiently promotes OFF-to-ON switching, while FimE primarily catalyses ON-to-OFF inversion (Abraham et al. 1985, Klemm 1986, Gally et al. 1996). The <i>fimI</i> gene was the last gene within the <i>fim</i> gene cluster to be characterized (Valenski et al. 2003). Within the <i>fim</i> gene cluster, there are 2 additional genes involved in transport and assembly of type 1 pili: <i>fimC</i> and <i>fimD</i>. The genes encoding tip fibrilum are <i>fimF</i>, <i>fimG</i>, <i>fimH</i>. Based on microarray gene expression studies by Schembri et al., performed with <i>E.coli</i> <i>K12</i> strain MG1655, it was found that the genes of the <i>fim</i> gene cluster are expressed in ratios of 100,
28, 8, 9, 4, 10 and 5 corresponding to genes \( \text{fimA, fimI, fimC, fimD, fimF, fimG and fimH} \), respectively (Schembri et al. 2002). The ratios were relative to \( \text{fimA} \), which was set to 100.

The biosynthesis and expression of P pili involves 11 genes encoded by the pyelonephritis associated pilus (pap) operon. \( \text{Pap} \) operon contains five structural genes \( \text{papA, papE, papF, papG, and papH} \), the usher gene \( \text{papC} \), the periplasmic chaperone gene \( \text{papD} \), and genes encoding two regulators, \( \text{papB} \) and \( \text{papI} \) (Soto and Hultgren 1999, Blomfield 2001) (Figure 5B). Expression of P fimbriae is regulated by phase variation, which is dependent on a reversible epigenetic switch that controls the initiation of transcription of the \( \text{pap} \) operon genes, resulting in active (phase “ON”) or inactive (phase “OFF”) expression of the structural subunits (Blyn et al. 1989, Blyn et al. 1990). The switch involves the formation of protein complexes on one of two GATC methylation sites located in the regulatory region (\( \text{papI-papB} \) intergenic region) upstream of the \( \text{papBA (PBA)} \) promoter. These GATC sites are methylation targets for DNA adenine methylase (Dam) and overlap two binding sites for the global regulator leucine-responsive regulatory protein (Lrp) (Braaten et al. 1991, Braaten et al. 1992). Competition between Dam and Lrp for access to these sites results in two different methylation patterns that determine whether the \( \text{pap} \) operon is transcriptionally “ON” or “OFF” (Vanderwoude et al. 1995, Weyand and Low 2000). Despite the very different molecular mechanisms controlling the expression of \( \text{pap} \) and \( \text{fim} \), the two systems share many features in common and have probably evolved to fulfill the same function.
Introduction

5A

**fim gene cluster**

Figure 5: Schematic overview of *fim* gene cluster and *pap* operon including the characterized promoter sites. 5A: *Fim* gene cluster is composed of 9 genes and one invertible element *fimS*. The regulatory genes *fimB* and *fimI* are shown in white. Downstream of invertible element *fimS* the genes *fimA* (cyan), *fimI* (grey), *fimC* (yellow) and *fimD* (blue) are encoding for main subunit FimA, termination subunit FimI, periplasmic chaperone FimC and usher FimD. Tip fibrilum components are encoding by genes *fimF* (brown), *fimG* (orange) and *fimH* (green), respectively. The expression of the *fim* genes is controlled by five promoters while in *pap* system only two promoters are required for expression of *pap* genes.

5B

**pap operon**

*Pap* operon consists of 11 genes: regulatory genes *papi* and *papB* shown in white, *papA* (cyan), *papH* (grey), *papC* (blue), *papD* (yellow), *papI* (white), *papK* (violet), *papE* (brown), *papF* (orange) and *papG* (green). Figures were created according to (Schwan 2011).
3.5. Goals of the thesis

The work presented here describes the final phase of type 1 pilus assembly, particularly the molecular mechanism of pilus growth termination, which has still not been fully understood. FimI is the subunit that has been proposed to terminate pilus assembly and to be required for anchoring the pilus to the outer membrane (Rossolini et al. 1993, Valenski et al. 2003). In the P pilus it is known that the minor subunits, PapH, is responsible for the termination of pilus growth by binding to the last unit of the main structural subunit, PapA, making up the helical pilus rod (Baga et al. 1987, Verger et al. 2006). Based on previous results in our laboratory we know that a similar mechanism is present in type 1 pili. The subunit FimI terminates the assembly of the pilus rod by irreversibly binding to the last incorporated main structural pilus subunit FimA on the periplasmic side of the usher pore thus preventing further incorporation of other pilus subunits. By the accumulation of pili in the medium rather than at the bacterial surface when expression of FimI was suppressed was shown that FimI functions as a molecular anchor (Ignatov 2009). Furthermore, in vitro data from our lab show that the pilus length is regulated by the FimI:FimA ratio and indicate that despite fimI and fimA being controlled by the same promoter and located in the same gene cluster, the periplasmic FimI concentration must be significantly lower compared to that of FimA (Ignatov 2009). Specifically, the FimD-catalyzed assembly of FimA in the presence of varying amounts of FimI showed that a natural pilus length distribution was only observed at an about 100-fold excess of FimA over FimI, while pilus rod formation was abolished at equimolar concentrations of FimA and FimI (Ignatov 2009). Therefore, the first goal was to find out how pilus length is regulated by the FimI:FimA ratio, why FimI is not present at similar concentrations as FimA and describe the different reasons why it might influence pilus biogenesis.

Since only subunits bound to chaperone FimC are assembly competent (Nishiyama et al. 2005), we speculated whether the lower periplasmic concentration of FimC-FimI compared to FimC-FimA might be due to inefficient binding of FimC to FimI or extremely slow oxidation process, what would lead to a “rare occurrence”. Therefore we tried to reveal the
mechanism of DsbA-dependent oxidation and FimC-catalyzed folding of FimI, investigate if FimI is able to fold without the help of FimC and provide more insights into termination process. The kinetic data would help to answer whether FimC-catalyzed folding of FimI plays a critical role on amounts of fully functional native FimI in the periplasm.

A further goal was to characterize the interactions between the FimC-FimI complex and the outer membrane usher FimD. For this purpose, the crystal structure of the FimC-FimI complex and the FimC-FimI-FimD<sub>NTD</sub> (N-terminal domain of FimD) were solved and analysed for specific features that could explain why pilus assembly stops once FimI has been incorporated.
4. RESULTS I: Biochemical and biophysical properties of FimI

4.1. Stability and refolding of FimI

First we tried to investigate whether FimI is able to fold without the help of FimC and reveal the mechanism of chaperone assisted folding of FimI. Since we speculated that the differences in FimC-catalyzed folding of FimA and FimI might be the reason for lower FimI abundance in the periplasm, we tried to purify full-length of FimI to could later compare it with the FimC-catalyzed full-length of FimA folding. We expressed FimI in the cytoplasm of E.coli BL21 (DE3) in form of inclusion bodies (Rudolph and Lilie 1996) (see chapter 7.5.1). To minimize formation of intramolecular disulfide bond, FimI was diluted to a final concentration of 2-5 μM and CuCl$_2$ at final concentration of 1 μM was added to catalyze formation of the single disulfide bond by air oxidation. Oxidation of FimI was performed at alkaline conditions in 6 M GdmCl, 20 mM Tris/HCl pH 8.0 mixing for 24-48 hours at 20 °C.

The formation of FimI’s disulfide bond was verified by Ellman’s test (Ellman 1959) and disulfide-intact unfolded FimI ($FimI^{U_{ox}}$) was further used for refolding. To test whether FimI can be refolded without the help of the chaperone FimC, first we tried 4 buffers with different pH-values (pH 5.0 - pH 8.0). The best refolding conditions were at pH 5.0 and pH 6.0. To further improve refolding of FimI, we used different additives: 0.5 M Arginine, 2 %, 5 % and 10 % Glycerol. Oxidized, unfolded $FimI^{U_{ox}}$ was refolded by rapid 30 x dilution and the final concentration of FimI after dilution was 5 μM. Refolding was performed for 2 hours at 4 °C. To remove additives, proteins were dialyzed against buffers (pH 5.0 - pH 8.0) at 4 °C overnight (o/n). After dialyzing, samples were centrifuged at 20 000 rpm, 30 min at 4 °C. Soluble (S- supernatant) and insoluble (P-pellet) fractions were analysed by SDS-PAGE, see Figure 6. The best refolding conditions, where $FimI^{N_{ox}}$ stayed soluble, were at pH 5.0 and pH 6.0. However $FimI^{N_{ox}}$ tends to slowly aggregate even if it is kept on ice. At pH 7.0 and pH 8.0, no significant amounts of soluble $FimI^{N_{ox}}$ were obtained. The additives, 0.5 M Arginine, 2 % and 5 % Glycerol, did not improve refolding at pH 5.0 and pH 6.0, whilst using 10 % Glycerol at pH 5.0 resulted in the same refolding conditions as seen on Figure 6.
corresponding to condition at pH 5.0 without additives. To test whether soluble FimI\textsubscript{N\text{ox}} obtained after refolding at pH 5.0 was folded, far- and near-UV CD spectra were measured, which report on secondary and tertiary structure of a protein, respectively (Figure 7). The far-UV CD spectrum revealed β-sheet content, which is characteristic for all pilus subunits. The negative signal (-5000 deg cm\textsuperscript{2} dmol\textsuperscript{-1}) with a minimum at 215 nm confirmed that FimI\textsubscript{N\text{ox}} possesses the secondary structure (Figure 7A). Near-UV CD spectra were measured for both refolded FimI\textsubscript{N\text{ox}} at pH 5.0 and unfolded FimI\textsubscript{U\text{ox}} in 3 M GdmCl at pH 5.0 (Figure 7B).

![Figure 6: Refolding of full-length of FimI in different pH conditions. FimI was refolded in 4 different buffer conditions: 20 mM Acetic acid/NaOH pH 5.0, 20 mM MES/NaOH pH 6.0, 20 mM MOPS/NaOH pH 7.0 and 20 mM Tris/HCl pH 8.0. 15 % SDS-PAGE gel shows the soluble fractions of FimI represented by a symbol (S)-supernatant and insoluble fraction by (P)-pellet. The black arrow is pointing to a band for FimI, whose position is consistent with low molecular weight marker (97.4, 66.2, 45, 31, 21.5 and 14.4 kDa).]

<table>
<thead>
<tr>
<th>pH 5.0</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
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<tr>
<td>S</td>
<td>P</td>
<td>S</td>
<td>P</td>
</tr>
<tr>
<td>97.4</td>
<td>66.2</td>
<td>45</td>
<td>31</td>
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</tbody>
</table>

![Figure 7: Spectroscopic characterization of FimI at pH 5.0, 25 °C. 7A: Far-UV CD spectrum of refolded FimI\textsubscript{N\text{ox}} at pH 5.0 was recorded at 200-250 nm, 25 °C and protein concentration used in this experiment was 0.1 mg/ml. 7B: Near-UV CD spectrum of refolded FimI\textsubscript{N\text{ox}} (black spectrum) and unfolded FimI\textsubscript{U\text{ox}} in 3 M GdmCl (blue spectrum). Spectra were recorded at 250-400 nm, 25 °C, protein concentration was 0.4 mg/ml.]

7A: [Graph of far-UV CD spectrum]
7B: [Graph of near-UV CD spectrum]
The maximum signal difference in near-UV CD is almost 2000 deg cm$^2$ dmol$^{-1}$ for the unfolded FimI$^U_{ox}$ (blue spectrum) in comparison to folded FimI$^N_{ox}$ (black spectrum). Since the shape of these spectra cannot be predicted, we can only speculate that this is a *fingerprint* of FimI. Next, we tested FimI$^N_{ox}$ for stability in different salt concentrations from 0 mM NaCl to 500 mM NaCl after 5 min incubation at pH 5.0, 25 °C (Figure 8). The emission spectra for 0 mM and 20 mM NaCl indicate that FimI$^N_{ox}$ remains unchanged after 5 min incubation. However, further increasing salt concentration applied on FimI$^N_{ox}$ caused that FimI$^N_{ox}$ aggregation increases with the concentration of NaCl (spectra 50 mM -500 mM NaCl).

![Figure 8: Stability of FimI$^N_{ox}$ against aggregation in different NaCl concentrations.](image)

Figure 8: Stability of FimI$^N_{ox}$ against aggregation in different NaCl concentrations. The refolded FimI$^N_{ox}$ was incubated in 6 different salt concentrations (0-500 mM NaCl) in 20 mM Acetic acid/NaOH pH 5.0, 25 °C for 5 min. After incubation, emission spectra were recorded at $\lambda_{ex}$: 280 nm. Protein concentration of FimI$^N_{ox}$ was 0.5 µM.

### 4.2. Time dependence of solubility of FimI$^N_{ox}$

To further characterize the stability of FimI$^N_{ox}$ against spontaneous aggregation, we measured time-dependent fluorescence emission spectra after transferring FimI$^N_{ox}$ from buffer at pH 5.0 to more physiological conditions at pH 7.0 and pH 8.0. We incubated refolded FimI$^N_{ox}$ after buffer exchange to 20 mM MOPS/NaOH pH 7.0 and 20 mM Tris/HCl pH 8.0 on ice for different time intervals (60, 90, 120 and 180 min). As a control we used non-incubated FimI$^N_{ox}$ (0 min). Figure 9A shows the emission fluorescence spectra recorded at $\lambda_{ex}$: 280 nm for a condition at pH 8.0 and Figure 9B for a condition at pH 7.0.
Since we did not observe any shift of the fluorescence intensity maximum to higher wavelength, we can exclude that FimI unfolds during incubation. The fluorescence intensity decrease by 15.4 % at pH 8.0 and 31 % at pH 7.0 after 60 min incubation on ice and unchanged intensity maxima at 340 m are showing that $\text{FimI}^\text{N}_{\text{ox}}$ aggregates 2 x faster at pH 7.0 compared to pH 8.0. The aggregation of $\text{FimI}^\text{N}_{\text{ox}}$ after 90, 120 and 180 min was observed by presence of white pellet after centrifugation of samples. Figure 9C illustrates fluorescence maxima at 340 nm obtained from individual emission spectra plotted against incubation time for both pHs, pH 7.0 (dots, color code same as in Figure 9A,B) and pH 8.0 (triangles, color code same as in Figure 9A,B). We showed that $\text{FimI}^\text{N}_{\text{ox}}$ stays more soluble at pH 8.0 than at pH 7.0 with a half-life of approximately 90 min.

Figure 9: Spectroscopic characterization of refolded $\text{FimI}^\text{N}_{\text{ox}}$ at pH 7.0 and 8.0, 25 °C. 9A: Fluorescence emission spectra of refolded $\text{FimI}^\text{N}_{\text{ox}}$ incubated at pH 8.0 after different time points. The color code of time intervals of 0, 60, 90, 120 and 180 min is black, blue, magenta, green and purple, respectively. The
protein concentration: 0.5 µM, \( \lambda_{ex} \): 280 nm.  

**9B:** Fluorescence emission spectra of refolded \( \text{FimI}^{\text{N}} \) incubated at pH 7.0. The protein concentration: 0.5 µM, \( \lambda_{ex} \): 280 nm and color code same as in Figure 9A. 

**9C:** The maximal signal change at 340 nm at pH 8.0 (Δ) and at pH 7.0 (○) were plotted against incubation time. The color code of time intervals is same as in Figure 9A and Figure 9B. All figures were generated in OriginePro9.

### 4.3. Tryptophan fluorescence increase upon oxidation of FimI

Here, we wanted to test whether association of FimI and FimC can be monitored by fluorescence spectroscopy. FimI is a monomeric one-domain protein (17.17 kDa), which possesses 3 tryptophans. One tryptophan is located at the beginning of the sequence W4 and two in the binding site for FimC, namely W154 and W121. FimC has 2 tryptophans, W36 and W84. As FimI undergoes DSC provided by FimC (Ignatov 2009), we could purify a complex of FimC-FimI. First, we tested at which concentration of GdmCl FimI dissociates from the FimC-FimI complex and unfolds. Figure 10 is showing fluorescence emission spectra of purified FimC-FimI complex at 0.5 µM incubated in three different GdmCl concentrations (0 M, 0.5 M and 3 M) for 5 min at RT. After centrifugation of samples, emission spectra were recorded at excitation wavelength 280 nm (\( \lambda_{ex} \)) and emission wavelength (\( \lambda_{em} \)) between 300-450 nm. The emission spectra showed that FimI stays still folded and bound to the complex FimC-FimI up to 0.5 M GdmCl.

![Fluorescence emission spectra](image-url)  

**Figure 10:** Fluorescence emission spectra documenting the dissociation of FimI from the FimC-FimI complex at pH 8.0, 25 °C. The spectra of purified FimC-FimI complex in different GdmCl concentrations (0, 0.5 and 3 M GdmCl) were recorded at \( \lambda_{ex} \): 280 nm, protein concentration: 0.5 µM.
To test whether binding of FimI to FimC can be detected via a tryptophan fluorescence change in FimC and FimI, we measured tryptophan fluorescence emission spectra of FimI\textsuperscript{N,ox}, FimI\textsuperscript{N,red}, FimC, purified FimC-FimI\textsuperscript{N,ox} and mixture of FimI\textsuperscript{U,ox} and FimC. The spectra were recorded at an excitation wavelength of 280 nm, emission 305-450 nm at final concentrations of 0.5 µM in 20 mM Tris/HCl pH 8.0 at 25 °C. The calculated sum of individual emission spectra under native conditions, refolded FimI\textsuperscript{N,ox} and folded FimC, is shown by solid black line (Figure 11). An individual spectrum of FimC is shown in green, spectrum of FimI\textsuperscript{N,ox} in purple and a mixture of FimI\textsuperscript{U,ox} with FimC (magenta solid line). Figure 11 shows a ~13 % increase in fluorescence intensity indicated by the upward-pointing black arrow. Based on this we can conclude that the tryptophan fluorescence change from binding FimC to FimI\textsuperscript{U,ox} and can clearly be detected by fluorescence spectroscopy and is characterized by tryptophan fluorescence increase.

![Figure 11: Fluorescence emission spectra of oxidized and reduced FimI at pH 8.0, 25 °C. Fluorescence emission spectrum of purified complex FimC-FimI\textsuperscript{N,ox} (blue line), spectrum of refolding FimI\textsuperscript{U,ox} at final concentration of 0.27 M GdmCl in presence of FimC (magenta line), sum of individual spectra FimC + FimI\textsuperscript{ox} (black line), FimC (green line), spectrum of refolded oxidized FimI\textsuperscript{ox} at 0.27 M GdmCl (purple line). Protein concentrations: 0.5 µM, λ\textsubscript{ex}; 280 nm. Figure was generated in OriginPro9 software.](image-url)
4.4. Disulfide bond in FimI is indispensable for FimC-FimI complex formation

Earlier observations showed that disulfide bond in FimA\textsubscript{U\text{red}} needs to be formed by DsbA\textsubscript{ox} and only then the binding of FimA\textsubscript{U\text{ox}} to FimC is possible, since FimA\textsubscript{U\text{red}} is not recognized by FimC (Crespo et al. 2012). Therefore to goal of this experiment was to test whether the same mechanism is true for FimI. To answer this we investigated binding of FimI\textsubscript{U\text{red}} and FimI\textsubscript{U\text{ox}} to FimC analysis by cation exchange chromatography. To keep FimI\textsubscript{U\text{red}} in 100 % reduced state, we tried to avoid air oxidation by filtrating and de-gassing solution containing EDTA. To insure that FimI is fully reduced, we determined the presence of free thiols in FimI\textsubscript{U\text{red}} by Ellman’s assay (Ellman 1959). All reactions were performed at pH 8.0, 4° C and detected at absorbance 230 nm. In Figure 12A, 2-fold molar excess of FimC (5 µM) over FimI\textsubscript{U\text{red}} (2.5 µM) was mixed and incubated for 5 min at RT. After incubation, DsbA\textsubscript{ox} (30 µM) was added into the same reaction and protein mixture was separated via analytical cation exchange chromatography. A small peak indicated by a downward pointing arrow corresponded to a formed FimC-FimI\textsuperscript{N\text{ox}} complex. As shown in Figure 12B, there is no peak for FimI alone confirming the fact that neither FimI\textsubscript{U\text{red}} nor FimI\textsubscript{U\text{ox}} after rapid dilution stays in stable state. A control reaction (5 µM FimC alone) is displayed in Figure 12C. After mixing 2-fold molar excess of FimC (5 µM) over FimI\textsubscript{U\text{ox}} (2.5 µM) (Figure 12D), an additional peak and decrease in the FimC peak intensity indicated complex formation of FimC-FimI\textsuperscript{N\text{ox}}. The intensity of the observed peak for FimC-FimI\textsuperscript{N\text{ox}} complex is comparable to peak intensity for FimC-FimI\textsuperscript{N\text{ox}} shown in Figure 12A (downward arrow). No peak was detected, when we mixed reduced form of FimI\textsubscript{U\text{red}} (2.5 µM) together with 2-fold molar excess of FimC (5 µM) (Figure 12E). Thus, the mechanism of oxidation of FimI is consistent with the oxidation mechanism shown for FimA\textsubscript{U\text{red}}, FimG\textsubscript{U\text{red}} and FimH\textsubscript{U\text{red}} pilus subunits (Crespo et al. 2012), where no recognition of unfolded reduced form of pilus subunits by FimC was detected. We were also speculating whether low periplasmic concentration of FimI compared to FimA can be a consequence of extremely slow oxidation of FimI\textsubscript{U\text{red}} catalysed by DsbA\textsubscript{ox} in comparison with other Fim subunits. To have more quantitative data about the oxidation mechanism of FimI subunit, we further performed stopped-flow tryptophan fluorescence kinetic experiments.
Figure 12: Disulfide bond formation in unfolded FimI is necessary for formation FimC-FimI complex.

12A: A reaction of 2-fold molar excess of FimC (5 µM) over FimI\textsubscript{U\text{red}} (2.5 µM) was first incubated together for 5 min at RT. To this reaction 12-fold molar excess of DsbA\textsubscript{ox} (30 µM) was added and incubated for further 5 min at RT. Protein mixture was then separated via analytical cation exchange column. The abundance of DsbA\textsubscript{ox} and DsbA\textsubscript{red} were observed in flow-through. Decrease in FimC peak intensity and formation of FimC-FimI peak (indicated downward arrow) are clear evidence that FimI\textsubscript{U\text{red}} was oxidized by DsbA and then FimC-FimI complex was formed. 12B: Both, FimI\textsubscript{U\text{ox}} and FimI\textsubscript{U\text{red}} were applied on a column at concentration of 2.5 µM and both were detected in a flow through. 12C: To see an elution profile of FimC alone, 5 µM of FimC was loaded on a column. 12D: In this reaction, a mixture of 2-fold molar excess of FimC (5 µM) over fully oxidized unfolded FimI\textsubscript{U\text{ox}} (2.5 µM) was incubated for 5 min at RT. Two peaks were observed, one corresponding to a complex formation of FimC-FimI (downwards arrow) and the second to free FimC. 12E: Reduced unfolded FimI\textsubscript{U\text{red}} (2.5 µM) was incubated with 2-fold molar excess of FimC (5 µM) for 5 min at RT. There is no peak evidence that FimC recognizes reduced form of FimI. All reactions were detected at 230 nm via an analytical cation exchange column RESOURCE S 1 ml at pH 8.0, RT.

The identity of the individual peaks and flow through fractions obtained in condition 12A were analysed by 15 % SDS-PAGE gel (Figure 13). The abundance of DsbA\textsubscript{ox}, reduced DsbA\textsubscript{red} upon oxidation of FimI and reduced FimI\textsubscript{U\text{red}} were detected in flow through fractions (lane 1-
3). The first peak eluted from a cation exchange column (elution volume ≈ 18 ml) is corresponding to free FimC (lanes 5-7) and second peak to a complex formation of FimC-FimI\textsuperscript{Nox} (lanes 9-11) (elution volume ≈ 24 ml).

![Flow through First peak Second peak](image)

**Figure 13**: The reaction of FimI\textsuperscript{U_red} in the presence of DsbA\textsuperscript{ox}/FimC analysed by 15% SDS-PAGE gel. Fractions loaded on the gel are corresponding to those observed in condition used in Figure 12A. The molecular masses of individual proteins are indicated on the right (FimC: 23.553 kDa, DsbA\textsuperscript{ox}: 21.132 kDa and FimI\textsuperscript{Nox}: 17.171 kDa). The black arrows are pointing to a band for FimC and for FimI\textsuperscript{Nox} whose positions are consistent with low molecular weight marker (97.4, 66.2, 45, 31, 21.5 and 14.4 kDa).

### 4.5. Oxidative folding of FimI\textsuperscript{U_red} by DsbA\textsuperscript{ox}

Bacteria express a wide range of disulfide-bonded virulence factors, including secreted toxins, surface components, such as adhesins and pili, and secretion systems (Lee et al. 2003). To be functionally active, many of these proteins must be oxidatively folded (Dutton et al. 2008). Several studies have demonstrated a direct role for periplasmic disulfide oxidoreductase, DsbA, in the biogenesis of virulence factors utilized by bacterial pathogens in various stages of the infection process (Heras et al. 2009). Therefore we used the termination structural subunit FimI to investigate the role of the disulfide bond of FimI in FimC–FimI complex formation and subunit folding. Since the typical rate for DsbA-catalyzed oxidation of small polypeptides at pH 7.0 is in the range of $10^5$-$10^7$ M\textsuperscript{-1}s\textsuperscript{-1} (Wunderlich et al. 1993) and recently was revealed that the rate constant for introducing disulfide bond into main pilus subunit of FimA yielded $1.4 \times 10^4$ M\textsuperscript{-1}s\textsuperscript{-1} at pH 7.0 (Crespo et al. 2012), we
performed kinetics experiments using the stopped-flow tryptophan fluorescence spectroscopy. To prevent strong fluorescence contributions from FimC, a spectroscopically silent his-tagged variant of FimC<sub>yy</sub>W36YW84Y (tryptophans W36 and W84 were replaced for tyrosines (Y), see chapter 7.5.2) was used. The tryptophan-free FimC variant FimC<sub>yy</sub> is fully functional and complements FimC deficiency in vivo (Vetsch et al. 2004). Since the stability of FimI is strongly influenced by pH (see chapter 4.2) all kinetics experiments were measured at pH 8.0. Figure 14A shows the tryptophan fluorescence increase due to the reduction of DsbA<sub>ox</sub> (Wunderlich and Glockshuber 1993) and oxidation of FimI<sup>U_red</sup>. The reactions were fitted globally according to a bimolecular reaction A+B→C. The oxidation reaction of FimI<sup>U_red</sup> (0.75 µM) by DsbA<sub>ox</sub> (5 µM) under the pseudo-first order conditions is displayed by black dots while a reaction at equimolar concentrations of FimI<sup>U_red</sup> and DsbA<sub>ox</sub> (0.75 µM) by grey dots. The obtained second-order rate constant was 1.15 x 10<sup>6</sup> M<sup>-1</sup>s<sup>-1</sup> (Figure 14A). No fluorescence signal change was detected after mixing FimI<sup>U_ox</sup> (0.75 µM) in > 5-fold molar excess of DsbA<sub>ox</sub> (5 µM). Figure 14B shows the tryptophan fluorescence increase due to complex formation upon refolding of FimI<sup>U_ox</sup> (0.75 µM) in > 5-fold molar excess of FimC<sub>yy</sub> (5 µM). The second-order rate constant for FimC<sub>yy</sub>-FimI<sup>N_ox</sup> (4.39 x 10<sup>4</sup> M<sup>-1</sup>s<sup>-1</sup>) is consistent with second-order rate constant obtained for FimC-FimI<sup>N_ox</sup> (4.05 x 10<sup>4</sup> M<sup>-1</sup>s<sup>-1</sup>) (Figure 14C). As was shown for FimA<sup>U_red</sup>, FimG<sup>U_red</sup> and FimH<sup>U_red</sup> (Crespo et al. 2012) and now also for FimI<sup>U_red</sup>, FimC is not able to recognize unfolded subunits missing the disulfide bond, therefore no fluorescence change was observed when FimC was mixed with FimI<sup>U_red</sup> (Figure 14C, grey dots). The kinetics of the combined oxidation and binding/folding reaction at initial concentrations of 5 µM DsbA<sub>ox</sub>, 5 µM FimC or FimC<sub>yy</sub> and 0.75 µM FimI<sup>U_red</sup> confirmed that DsbA-dependent oxidation of FimI<sup>U_red</sup> (k = 1.15 x 10<sup>6</sup> M<sup>-1</sup>s<sup>-1</sup>) proceeded 25 x faster than FimC-catalyzed folding of FimI<sup>U_ox</sup> (k = 4.05 x 10<sup>4</sup> M<sup>-1</sup>s<sup>-1</sup>) (Figure 14D and Figure 14E). The fluorescence change was analysed according to two consecutive, irreversible steps (see chapter 7.13; Figure 14D and 14E) and the obtained rate constants were identical within experimental error to those obtained for the individual reactions (Figure 14A, 14B, 14C). To conclude our kinetic data, we showed that only unfolded oxidized FimI<sup>U_ox</sup> is able to bind FimC/FimC<sub>yy</sub>, what also ruins the hypothesis that the termination of type 1 pilus occurs due to inefficient FimC-catalyzed folding of FimI. Moreover, the kinetic data presented here
show efficient and fast oxidation of FimI\textsubscript{U red} by DsbA\textsubscript{ox} and a slightly faster rate constant of FimC-catalyzed folding of FimI\textsubscript{U ox} ($k = 4.05 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) compared to FimC-catalyzed folding of the main structural subunit FimA ($k = 2.83 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) (Crespo et al. 2012).
Figure 14: Stopped-flow fluorescence kinetics of oxidation FimI\textsuperscript{U}\textsubscript{red} by DsbA\textsubscript{ox} followed by refolding of FimI\textsuperscript{U}\textsubscript{ox} in molar excess of FimC/FimC\textsubscript{Wy} (pH 8.0, 25 °C) 14A: The green solid line corresponds to a global fit describing a bimolecular reaction (A+B→C) with fixed concentrations of FimI, DsbA\textsubscript{ox} and a shared rate constant “k\textsuperscript{t}” using a normalized second-order equation. Oxidation of FimI\textsuperscript{U}\textsubscript{red} (0.75 μM) by DsbA\textsubscript{ox} (0.75 μM) at equimolar concentrations is shown by grey dots and a reaction with > 5-fold molar excess of DsbA\textsubscript{ox} over FimI by black dots. 14B: Tryptophan fluorescence increase due to a complex formation upon refolding of FimI\textsuperscript{U}\textsubscript{ox} (0.75 μM) with > 5-fold molar excess of FimC\textsubscript{Wy} (5 μM). The green solid line corresponds to a fit according to pseudo-first-order reaction and symbol k\textsubscript{1} corresponds to a rate constant for a complex formation FimC\textsubscript{Wy}→FimI\textsuperscript{N}\textsubscript{ox}. 14C: The individual reaction of refolding FimI\textsuperscript{U}\textsubscript{ox} (0.75 μM) in an excess of FimC (5 μM FimC) was analysed according to a pseudo-first-order reaction. No fluorescence change was observed when FimI\textsuperscript{U}\textsubscript{red} (0.75 μM) was mixed with 5 μM FimC (grey dotted trace). 14D: Tryptophan fluorescence kinetics of the combined oxidation of FimI\textsuperscript{U}\textsubscript{red} by DsbA and FimC\textsubscript{Wy} catalyzed binding/folding of FimI\textsuperscript{U}\textsubscript{ox} at final concentrations of 5 μM DsbA\textsubscript{ox}, 5 μM FimC\textsubscript{Wy} and 0.75 μM FimI\textsuperscript{U}\textsubscript{red}. The solid green line corresponds to a fit according to two consecutive irreversible reactions (A→B→C) yielding two pseudo-first order rate constants (k\textsubscript{1} and k\textsubscript{2}). Reaction was fitted using program Berkeley Madonna 8.3 (Macey & Oster) (see equation, chapter 7.13). 14E: Combined oxidation of 0.75 μM FimI\textsuperscript{U}\textsubscript{red} by 5 μM DsbA\textsubscript{ox} and refolding of FimI\textsuperscript{U}\textsubscript{ox} with > 5-fold molar excess of FimC (5 μM). The trace was fitted according to two consecutive irreversible steps (A→B→C) yielding 2 pseudo-first order rate constants k\textsubscript{1} and k\textsubscript{2}. All rate constants are summarized in Table 1 and all plots were generated in program Origin Pro9.

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Conditions</th>
<th>k\textsubscript{1} (Oxidation) (M\textsuperscript{-1}s\textsuperscript{-1})</th>
<th>k\textsubscript{2} (Folding) (M\textsuperscript{-1}s\textsuperscript{-1})</th>
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<tbody>
<tr>
<td>14A</td>
<td>0.75 μM FimI\textsuperscript{U}\textsubscript{red} + 0.75 μM DsbA\textsubscript{ox}</td>
<td>(1.15 ± 0.01) x 10\textsuperscript{6}</td>
<td>-</td>
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<tr>
<td></td>
<td>0.75 μM FimI\textsuperscript{U}\textsubscript{red} + 5 μM DsbA\textsubscript{ox}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14B</td>
<td>0.75 μM FimI\textsuperscript{U}\textsubscript{red} + 5 μM FimC\textsubscript{Wy}</td>
<td>(4.39 ± 0.03) x 10\textsuperscript{6}</td>
<td>-</td>
</tr>
<tr>
<td>14C</td>
<td>0.75 μM FimI\textsuperscript{U}\textsubscript{red} + 5 μM FimC</td>
<td>(4.05 ± 0.01) x 10\textsuperscript{4}</td>
<td>-</td>
</tr>
<tr>
<td>14D</td>
<td>0.75 μM FimI\textsuperscript{U}\textsubscript{red} + 5 μM DsbA\textsubscript{ox} + 5 μM FimC\textsubscript{Wy}</td>
<td>(4.16 ± 0.03) x 10\textsuperscript{4}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(fixed rate)</td>
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<td>-</td>
</tr>
<tr>
<td>14E</td>
<td>0.75 μM FimI\textsuperscript{U}\textsubscript{red} + 5 μM DsbA\textsubscript{ox} + 5 μM FimC</td>
<td>(1.26 ± 0.08) x 10\textsuperscript{6}</td>
<td>4.05 x 10\textsuperscript{4}</td>
</tr>
<tr>
<td></td>
<td>(fixed rate)</td>
<td>-</td>
<td>-</td>
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</table>

Table 1: The summary of all rate constants of oxidation and folding of FimI by DsbA and FimC.
4.6. The pilus subunit FimI folds as fast as the main subunit FimA

To compare oxidation of $\text{FimI}^{\text{U, red}}$ by $\text{DsbA}_{\text{ox}}$ and FimC-catalyzed folding of full-length FimI with that of full-length FimA at pH 8.0, 25 °C, we measured stopped-flow fluorescence kinetics of oxidation of $\text{FimA}^{\text{U, red}}$ by $\text{DsbA}_{\text{ox}}$. The second-order rate constant yielded for the oxidation of $\text{FimA}^{\text{U, red}}$ by $\text{DsbA}_{\text{ox}}$ was $(3.38 \times 10^4 \text{ M}^{-1}\text{s}^{-1})$ (Figure 15A) what is 30 times more slow than the oxidation of FimI $(1.15 \times 10^6 \text{ M}^{-1}\text{s}^{-1})$ (Figure 14A). However, the explanation why FimI$^{\text{U, red}}$ is oxidized significantly faster than FimA$^{\text{U, red}}$ is unclear. Next, we measured the individual reaction for FimC-catalyzed folding of FimA$^{\text{U, ox}}$ what yielded the second-order rate constant $(4.28 \times 10^4 \text{ M}^{-1}\text{s}^{-1})$ (Figure 15B). As shown in Figure 15A oxidation is characterized by increasing tryptophan fluorescence signal whereas the folding/binding of FimA by FimC has characteristic decreasing fluorescence signal (Figure 15B). The DsbA-dependent oxidation of FimA$^{\text{U, red}}$ and FimI$^{\text{U, red}}$ occurs then identical, characterized by tryptophan fluorescence increase in comparision to the complex formation of FimC-FimA$^{\text{N,ox}}$ (fluorescence decrease) and FimC-FimI$^{\text{N,ox}}$ (fluorescence increase). Figure 15C describes the kinetics of coupled oxidation of FimA$^{\text{U, red}}$ and subsequent binding/folding of FimA$^{\text{U, ox}}$ on a surface of FimC. The yielded second-order rate constants are consistent to those obtained from the individual reactions. By showing identical rate constants for FimC-FimI$^{\text{N,ox}}$ and FimC-FimA$^{\text{N,ox}}$, we ruined the hypothesis that the low FimI concentration in periplasm is due to extremely slow or inefficient FimC-catalyzed folding of FimI. Although FimI and FimA have very different functions in type 1 pilus biogenesis they fold with the same rate.

\begin{align*}
\text{FimA}^{\text{U, red}} + \text{DsbA}_{\text{ox}} &\rightarrow \text{FimA}^{\text{U, ox}} + \text{DsbA}_{\text{red}} \\
\text{FimA}^{\text{U, ox}} + \text{FimC} &\rightarrow \text{FimC-FimA}^{\text{N, ox}}
\end{align*}

\begin{align*}
k_1 &= (3.38 \pm 0.01) \times 10^4 \text{ M}^{-1}\text{s}^{-1} \\
[F\text{imA}^{\text{U, ox}}] &= 0.75 \mu\text{M} + [\text{DsbA}_{\text{ox}}] = 5\mu\text{M} \\
[F\text{imA}^{\text{U, red}}] &= 0.75 \mu\text{M} + [\text{DsbA}_{\text{ox}}] = 5\mu\text{M} \\
k_2 &= (4.28 \pm 0.15) \times 10^4 \text{ M}^{-1}\text{s}^{-1} \\
[F\text{imA}^{\text{U, ox}}] &= 0.75 \mu\text{M} + [\text{FimC}] = 5\mu\text{M}
\end{align*}
Figure 15: Stopped-flow fluorescence kinetics of oxidation of FimA\textsuperscript{U}\textsubscript{red} by DsbA\textsubscript{ox} and subsequent binding/folding of FimA\textsuperscript{U}\textsubscript{ox} on a surface of FimC (pH 8.0, 25 °C). 15A: The tryptophan fluorescence increase is due to the reduction of DsbA\textsubscript{ox} (5 μM) and oxidation of FimA\textsuperscript{U}\textsubscript{red} (0.75 μM). The green solid line corresponds to a fit according to a pseudo-first-order reaction and symbol k\textsubscript{1} to an obtained rate constant for DsbA-dependent oxidation of FimA\textsuperscript{U}\textsubscript{red}. No reaction occurs when FimA\textsuperscript{U}\textsubscript{red} is mixed with DsbA\textsubscript{ox} (grey trace). 15B: Tryptophan fluorescence decrease of FimC upon binding of FimA to FimC and DsbA, and formation of FimC-FimA\textsuperscript{N}\textsubscript{ox}. Reaction was fitted according to pseudo-first-order reaction indicated by a green solid line and symbol k\textsubscript{2} corresponds to a rate constant for the complex formation FimC-FimA\textsuperscript{N}\textsubscript{ox}. 15C: The tryptophan fluorescence kinetics of the combined DsbA-dependent oxidation and FimC-catalyzed binding/folding of FimA at final concentrations of 0.75 μM FimA\textsuperscript{U}\textsubscript{red}, 5 μM FimC and 5 μM DsbA\textsubscript{ox}. The tryptophan fluorescence increase is followed by fluorescence decrease due to the complex formation of FimC-FimA after mixing FimA\textsuperscript{U}\textsubscript{red} (0.75 μM) with more than 5-fold molar excess of FimC (5 μM) over FimA\textsuperscript{U}\textsubscript{red} (2.5 μM). The green solid line represents a fit according to two consecutive irreversible reactions (see equation described in 7.13). All rate constants are summarized in Table 2 and all plots were generated in program OriginPro9.

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Conditions</th>
<th>Mechanism</th>
<th>( k_1 ) (oxidation) (M\textsuperscript{-1}s\textsuperscript{-1})</th>
<th>( k_2 ) (folding) (M\textsuperscript{-1}s\textsuperscript{-1})</th>
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<tbody>
<tr>
<td>15A</td>
<td>0.75 μM FimA\textsuperscript{U}\textsubscript{red} + 5 μM DsbA\textsubscript{ox}</td>
<td>( A^{\text{U}\textsubscript{red}} \rightarrow A^{\text{U}\textsubscript{ox}} )</td>
<td>( 3.38 \pm 0.01 \times 10^4 )</td>
<td>-</td>
</tr>
<tr>
<td>15B</td>
<td>0.75 μM FimA\textsuperscript{U}\textsubscript{ox} + 5 μM FimC</td>
<td>( A^{\text{U}\textsubscript{ox}} \rightarrow A^{\text{N}\textsubscript{C}} )</td>
<td>-</td>
<td>( 4.28 \pm 0.15 \times 10^4 )</td>
</tr>
<tr>
<td>15C</td>
<td>0.75 μM FimA\textsuperscript{U}\textsubscript{red} + 5 μM DsbA\textsubscript{ox} + 5 μM FimC</td>
<td>( A^{\text{U}\textsubscript{red}} \rightarrow A^{\text{U}\textsubscript{ox}} \rightarrow A^{\text{N}\textsubscript{C}} )</td>
<td>( 3.4 \pm 0.01 \times 10^4 )</td>
<td>( 4.4 \pm 0.01 \times 10^4 )</td>
</tr>
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</table>

Table 2: Obtained rate constants of DsbA-dependent oxidation of FimA\textsuperscript{U}\textsubscript{red} and FimC-catalyzed complex formation of FimC-FimA\textsuperscript{N}\textsubscript{ox} at pH 8.0, 25 °C. Identical rate constant of FimA\textsuperscript{U}\textsubscript{red} oxidation by DsbA\textsubscript{ox} in an individual reaction (3.38 ± 0.01) \times 10^4 M\textsuperscript{-1}s\textsuperscript{-1}, Figure 15A was observed also in combined reaction shown in Figure 15C (3.4 ± 0.01) \times 10^4 M\textsuperscript{-1}s\textsuperscript{-1}. The kinetics of an individual reaction of FimC-FimA\textsuperscript{N}\textsubscript{ox} complex formation described in Figure 15B (4.28 ± 0.15) \times 10^4 M\textsuperscript{-1}s\textsuperscript{-1} yielded the same second-order rate constant as in Figure 15C (4.4 ± 0.01) \times 10^4 M\textsuperscript{-1}s\textsuperscript{-1}.

To simulate oxidative folding of FimI and FimA \textit{in vivo}, we used the experimentally obtained rate constants of FimI and FimA oxidation and binding to FimC at pH 8.0, 25 °C (see chapters 4.5 and 4.6, Table 1, 2). Based on the known periplasmic concentrations of DsbA\textsubscript{ox} (= 86 μM)
and FimC (= 23 µM) (Crespo et al. 2012), we predicted the in vivo half-life of oxidative folding of FimI and FimA in the periplasm. The kinetics of in vivo FimC-catalyzed folding of FimI and FimA were simulated using an equation as described in 7.13, where \( k_{1\text{app}} = k_1^* [\text{DsbA}_{\text{ox}}] \) and \( k_{2\text{app}} = k_2^* [\text{FimC}] \). The following equations describe the fractions of A, B, C (\( \gamma_A, \gamma_B, \gamma_C \)) representing FimI\(_{\text{U red}}\), FimI\(_{\text{U ox}}\), FimC-FimI\(_{\text{N ox}}\) at any time of the reaction as a function of \( k_1, k_2 \) and reaction time \( t \). Therefore based on the observed fractions \( F_{\text{obs}} \) for A, B and C, we were able to predict in vivo half-life independently on knowing the periplasmic concentration of FimI. Figure 16A illustrates the consecutive mechanism of DsbA- and FimC-catalyzed in vivo folding of FimI and FimA (Figure 16B). To compare in vivo and in vitro oxidative folding of FimI, the initial concentrations of 5 µM DsbA\(_{\text{ox}}\) and 5 µM FimC were taken into account. Disappearance of FimI\(_{\text{U red}}\) is very rapid step what leaded to almost 95 % accumulation of FimI\(_{\text{U ox}}\). Here, we showed faster in vivo half-life of FimI (0.7 s) in comparison with in vivo half-life FimA (1.08 s) under the same conditions. Recently was shown that in vivo oxidative folding of FimA is faster than in vitro experiments measured at pH 7.0, 25 °C (Crespo et al. 2012). The in vitro experiment of oxidation of FimI\(_{\text{U red}}\) by DsbA (see Figure 14A) showed that the reaction is completed within less than 2 seconds what is slower than in vivo oxidative folding of FimI at pH 8.0, 25 °C (0.7s, Figure 16A). Thus, FimI can play a role in type 1 pili biogenesis only after DsbA-catalyzed oxidation as it was shown for the other pilus subunits (Crespo et al. 2012).
Figure 16: Predicted in vivo folding kinetics of FimI and FimA. Both reactions are simulated according to 2 consecutive irreversible reactions with known periplasmic concentrations of DsbA_{ox} [≈ 86 µM] and FimC [≈23 µM] as shown in cartoon scheme. On graph 16A fractions A, B, C representing FimI_{red}, FimI_{ox}, FimC-FimI_{N ox} are plotted against reaction time (s). The reduced FimI_{red} is described by decreasing solid blue line, FimI_{ox} by a solid green line, FimC-FimI_{N ox} complex by an increasing solid black line. Figure 16B represents a graph of fractions of FimA_{red} (solid red line), FimA_{ox} (solid blue line), FimC-FimA_{N ox} (solid black line) plotted against reaction time. Both reactions were simulated by Berkeley Madonna 8.3 program (Macey & Oster).
4.7. Confirmation of the folding rates of FimA, FimI with a kinetic competition assay

Here, we experimentally tested the obtained rate constants for oxidative folding of Fim$\text{I}_{\text{red}}$ and Fim$\text{A}_{\text{red}}$ by Dsb$\text{A}_{\text{ox}}$ and FimC-catalyzed complex formation of FimC-Fim$\text{I}_{\text{ox}}$ and FimC-Fim$\text{A}_{\text{ox}}$ at pH 8.0 by analytical cation exchange chromatography at 4 °C. To predict how much complex of FimC-Fim$\text{I}_{\text{ox}}$ and FimC-Fim$\text{A}_{\text{ox}}$ is formed, we simulated following reactions: Fim$\text{A}_{\text{red}}$ + Dsb$\text{A}_{\text{ox}}$ → Fim$\text{A}_{\text{ox}}$ + Dsb$\text{A}_{\text{red}}$ (corresponding to: A+B → C+D), Fim$\text{A}_{\text{ox}}$ + FimC → FimC-Fim$\text{A}_{\text{ox}}$(C+E→F), Fim$\text{I}_{\text{red}}$ + Dsb$\text{A}_{\text{ox}}$ → Fim$\text{I}_{\text{ox}}$ + Dsb$\text{A}_{\text{red}}$ (G+B→H+D) and Fim$\text{I}_{\text{ox}}$ + FimC→ FimC-Fim$\text{I}_{\text{ox}}$(H+E→I). Figure 17A, 17B and 17C illustrate fractions of FimC-Fim$\text{A}_{\text{ox}}$ represented by a black solid line and fractions of FimC-Fim$\text{I}_{\text{ox}}$ by a green solid line. All reactions were simulated using Berkeley Madonna 8.3 program (Macey & Oster) by applying varied initial concentrations of reactants. First we simulated a reaction with initial concentrations (Fim$\text{I}_{\text{red}}$, Fim$\text{A}_{\text{red}}$, Fim$\text{C}$) equal to 1 µM and Dsb$\text{A}_{\text{ox}}$ to 2 µM, see Figure 17A. Figure 17B describes a reaction with initial concentrations of (Fim$\text{I}_{\text{red}}$, Fim$\text{A}_{\text{red}}$, Dsb$\text{A}_{\text{ox}}$, Fim$\text{C}$) equal to 1 µM and Fim$\text{C}$ to 2 µM. In a last reaction we simulated a 5-fold molar excess of Fim$\text{C}$ and Dsb$\text{A}_{\text{ox}}$(5 µM) over (Fim$\text{I}_{\text{red}}$, Fim$\text{A}_{\text{red}}$) (1 µM).
17C

Figure 17: Predicted fractions of native FimC-subunit complexes. 17A: First simulated reaction: FimA\textsubscript{U,red} + DsbA\textsubscript{ox} → FimA\textsuperscript{U,ox} + DsbA\textsubscript{red} (A+B→C+D), in which we used the experimentally obtained second-order-rate constant of FimA\textsubscript{U,red} oxidation by DsbA\textsubscript{ox} (3.38 ± 0.01) x 10\textsuperscript{4} M\textsuperscript{-1}s\textsuperscript{-1} with initial concentrations [A]=1 µM FimA\textsubscript{U,red}, [B]=2 µM DsbA\textsubscript{ox}. Second simulated reaction: FimC + FimA\textsubscript{U,ox} → FimC-FimA\textsuperscript{N,ox} (C+E→F) using experimentally obtained second-order-rate constant of FimC-catalyzed FimA folding and complex formation (4.4 ± 0.01) x 10\textsuperscript{4} M\textsuperscript{-1}s\textsuperscript{-1} with initial concentration [E]=1 µM FimC. Third simulated reaction: FimI\textsubscript{red} + DsbA\textsubscript{ox} → FimI\textsuperscript{ox} + DsbA\textsubscript{red} (G+B→H+D), in which k=1.15 x 10\textsuperscript{6} M\textsuperscript{-1}s\textsuperscript{-1} with initial concentrations [G]=1 µM FimI\textsubscript{red} and fourth simulated reaction: FimI\textsubscript{red} + FimC → FimC-Fim\textsuperscript{N,ox} (H+E→I), in which k= 4.05 x 10\textsuperscript{4} M\textsuperscript{-1}s\textsuperscript{-1} with initial concentrations [E]=1 µM FimC. Predicted complex formation of FimC-Fim\textsuperscript{N,ox} (35 %) and FimC-Fim\textsuperscript{N,ox} (65 %). 17B: Simulating reaction if concentration of (FimI\textsubscript{U,red}, FimA\textsubscript{U,red}, DsbA\textsubscript{ox}) is 1 µM and FimC is 2 µM. Predicted complex formation of FimC-Fim\textsuperscript{N,ox} (10 %) and FimC-Fim\textsuperscript{N,ox} (90 %). 17C: Simulating reaction if concentration of (FimI\textsubscript{U,red}, FimA\textsubscript{U,red}) is 1 µM and (FimC, DsbA\textsubscript{ox}) is 5 µM. Predicted complex formation of FimC-Fim\textsuperscript{N,ox} (100 %) and FimC-Fim\textsuperscript{N,ox} (100 %). All reactions were simulated using Berkeley Madonna 8.3 program (Macey & Oster).

To test all three simulated reactions experimentally, we mixed FimI\textsubscript{U,red} with FimA\textsubscript{U,red} together with a mixture of DsbA\textsubscript{ox}/FimC and incubated it for 5 min at RT. Formed complexes were then separated by cation exchange chromatography at 4 °C. In all reactions (Figure 18A, B, C) 5 different species were detected: the abundance of free FimC, formation of FimC-Fim\textsuperscript{N,ox} complex, dimmers of FimC-Fim\textsuperscript{N,ox}-Fim\textsuperscript{N,ox}, complex of FimC-Fim\textsuperscript{N,ox} and dimmers of FimC-Fim\textsuperscript{N,ox}-Fim\textsuperscript{N,ox} within 5 min of incubation. The percentage of formed complexes, FimC-Fim\textsuperscript{N,ox} and FimC-Fim\textsuperscript{N,ox}, were determined based on calculation of only FimC bound in a FimC-subunit in a FimC-subunit-subunit complexes at 230 nm absorbance. In a condition A, measured percentage for complex formation of FimC-FimA was 33.8 % and FimC-FimI 66.3 % compared to predicted 35 % and 65 %. In a condition B, obtained percentage by cation exchange chromatography for FimC-Fim\textsuperscript{N,ox} complex was 10 % and for FimC-Fim\textsuperscript{N,ox} 90 % compared to predicted 13.6 % and 86.4 %. In a condition C, calculated percentage for FimC-
FimA\textsuperscript{ox} complex was 100 % and for FimC-FimI\textsuperscript{ox} 100 % compared to predicted 54 % and 46 %.

**Results**

**Figure 18:** Experimentally confirmed simulations. Analytical, fast ion-exchange chromatography using RESOURCE S 1 ml: Condition A shows the result, if concentration of (FimI\textsubscript{red}, FimA\textsubscript{red}, FimC) is 1 µM and DsbA\textsubscript{ox} 2 µM. Complex formation of FimC-FimA\textsuperscript{ox} and FimC-FimI\textsuperscript{ox} was 33.8 % and 66.3 %, respectively. Condition B shows the result for concentrations of (FimI\textsubscript{red}, FimA\textsubscript{red}, DsbA\textsubscript{ox}) of 1 µM and FimC 2 µM. Complex formation of FimC-FimA\textsuperscript{ox} was 13.6 % and of FimC-FimI\textsuperscript{ox} 86.4 %. Condition C shows the result for concentrations of (FimI\textsubscript{red}, FimA\textsubscript{red}) of 1 µM and (FimC, DsbA\textsubscript{ox}) of 5 µM. Complex formation of FimC-FimA\textsuperscript{ox} was 54 % and FimC-FimI\textsuperscript{ox} was 46 %. Condition D shows formation of FimC-FimI\textsuperscript{ox} complex alone, condition E FimC alone and condition F complex FimC-FimA\textsuperscript{ox} alone. Peak areas of the individual peaks were determined by integration using Peak Fit SOFTWARE and chromatograms were illustrated by Sigma Plot 12.2 software.

4.8. The long N-terminal extension of FimI slows down FimI folding

4.8.1. FimC-catalyzed folding of FimI\textsubscript{ox}

Here we tried to understand the uniqueness of the longest donor strand of FimI among all other pili subunits of type 1 pili in uropathogenic *E.coli*. As revealed recently by Walczak and
Puorger (Walczak et al. 2014), the donor-strand of FimI is pseudo-palindromic (G^{11}N^{12}M^{13}Q^{14}F^{15}Q^{16}G^{17}). Two glycine residues (G^{11} and G^{17}) separated by five residues (N^{12}M^{13}Q^{14}F^{15}Q^{16}) have also been shown in natural donor strand of FimA subunit of uropathogenic *E. coli* and in many other pathogenic enterobacteria (Walczak et al. 2014). The unusual pseudo-palindromic sequence of FimI is defined by glycine residues (G^{11}, G^{17}) and Q^{14} which is the equivalent to His^{11} in FimA donor-strand sequence (Walczak et al. 2014).

The first approach was to measure stopped-flow tryptophan fluorescence kinetics of FimC-catalyzed folding of truncated variant of FimI (FimI_{Δ1-20}), namely FimI_{t}. FimI_{t} lacks the first 20 N-terminal amino acid residues. The tryptophan fluorescence change was recorded at the excitation wavelength of 280 nm, using a glass filter 320 nm at pH 8.0 and 25 °C. Fully oxidized denatured FimI_{tU_{ox}} was diluted into refolding buffer containing chaperone FimC in asymmetric mixing conditions (1:11) for pseudo-first order kinetic (Figure 19A) and in symmetric conditions (1:1) for equimolar concentrations (Figure 19B). The final concentration of remaining GdmCl after mixing was 0.27 M. Figure 19A illustrates pseudo-first order reactions with > 5-fold molar excess of FimC over FimI_{tU_{ox}}. The kinetics traces show tryptophan fluorescence change in FimC and FimI_{t} upon binding and folding of FimI_{t} on the surface of FimC. Reactions were fitted globally according to a bimolecular reaction A+B→C, where [A] ≠ [B] using OriginePro9. The yielded second-order rate constant was k = (3.43 ± 0.03) x 10^{5} M^{-1} s^{-1} (Figure 19A). Next, we measured the stopped-flow fluorescence kinetic of FimC-catalyzed folding of FimI_{tU_{ox}} in the presence of equimolar amounts of FimC and FimI_{tU_{ox}} (Figure 19B) under the same conditions as described in Figure 19A (fully oxidized FimI_{tU_{ox}} 0.27 M remaining GdmCl, pH 8.0, 25 °C). Data were fitted globally according to a bimolecular reaction A+B→C, where [A] = [B] using OriginePro9. The yielded second-order rate constant was k = (3.49 ± 0.01) x 10^{5} M^{-1} s^{-1} what is fully consistent with a second-order rate constant obtained in Figure 19A. Initial concentrations, mechanism and obtained rate constants are summarized in Table 3. Based on kinetics results measured under the pseudo-first order conditions and at equimolar concentrations by stopped-flow techniques, we determined the exact rate constant of refolding FimI_{tU_{ox}} by chaperone FimC. Yielded rate constants in Figure 19A and Figure 19B are faster than the observed rate constant of FimC-
catalyzed folding of FimI \((4.05 \pm 0.02) \times 10^4 \text{M}^{-1}\text{s}^{-1}\) (Figure 14B, 14C) confirming that N-terminal part of FimI slows down FimI subunit folding by one order of magnitude.

19A

![Graph showing stopped-flow kinetics of FimC-catalyzed folding of FimI at pH 8.0, 25 °C. All reactions were done under the pseudo-first order conditions with > 5-fold molar excess of FimC over FimI at 25 °C and pH 8.0. Kinetics of FimC-catalyzed folding of FimI\(_{\text{ox}}\) at different total protein concentrations: (0.5 \text{µM FimI}_{\text{ox}} and 2.5 \text{µM FimC- solid grey symbol}) (0.75 \text{µM FimI}_{\text{ox}} and 5 \text{µM FimC- solid violet symbol}) (2 \text{µM FimI}_{\text{ox}} and 10 \text{µM FimC-solid light blue}), (5 \text{µM FimI}_{\text{ox}} and 25 \text{µM FimC-dark green symbol}). The signals were normalized to the fractions of FimC-FimI complex. Each reaction of refolding FimI by FimC was recorded for 10 seconds. Datasets of all 4 kinetics reactions were fitted globally using a second-order fit (black solid lines) with fixed concentrations of FimI and FimC and shared rate constant “k” using a...](image)
normalized equation. The yielded second order rate constant, described by symbol \( k' \) was \((3.43 \pm 0.03) \times 10^5 \text{ M}^{-1} \text{s}^{-1}\). **19B:** Stopped-flow tryptophan fluorescence kinetics of FimC-catalyzed folding of FimI\(_{\text{ox}}^U\) at equimolar concentrations of FimC and FimI\(_{\text{ox}}^U\). Black solid line corresponds to a fit according to a second-order reaction \( A + B \rightarrow C \), where \([A] = [B]\). Both figures displaying kinetics traces were generated in OriginPro9 program.

<table>
<thead>
<tr>
<th>Figure</th>
<th>Conditions</th>
<th>mechanism</th>
<th>( k ) (M(^{-1})s(^{-1}))</th>
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<tbody>
<tr>
<td>19A</td>
<td><em>Pseudo-first order</em></td>
<td>( I_{\text{ox}}^U + C \rightarrow I_{\text{ox}}^C )</td>
<td>((3.43 \pm 0.05) \times 10^5)</td>
</tr>
<tr>
<td></td>
<td>0.5 µM FimI(_{\text{ox}}^U) + 2.5 µM FimC</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.75 µM FimI(_{\text{ox}}^U) + 5 µM FimC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 µM FimI(_{\text{ox}}^U) + 10 µM FimC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 µM FimI(_{\text{ox}}^U) + 25 µM FimC</td>
<td></td>
<td></td>
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<tr>
<td>19B</td>
<td><em>Equimolar concentrations</em></td>
<td>( I_{\text{ox}}^U + C \rightarrow I_{\text{ox}}^C )</td>
<td>((3.49 \pm 0.02) \times 10^5)</td>
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<tr>
<td></td>
<td>2 µM FimI(_{\text{ox}}^U) + 2 µM FimC</td>
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<tr>
<td></td>
<td>5 µM FimI(_{\text{ox}}^U) + 5 µM FimC</td>
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**Table 3:** Summarized rate constants of FimC-catalyzed folding of FimI\(_{\text{ox}}^U\) at pH 8.0, 25 °C. **19A:** Refolding kinetics of FimI\(_{\text{ox}}^U\) by FimC in four different concentrations with > 5-fold molar excess of FimC over FimI\(_{\text{ox}}^U\), obtained by stopped-flow fluorescence spectroscopy at 0.27 M GdmCl (\( \lambda_{\text{ex}}: 280 \text{ nm, } \lambda_{\text{em}}: >320 \text{ nm} \)). **19B:** Kinetics of FimC-catalyzed folding of FimI\(_{\text{ox}}^U\) at equimolar concentrations: 2 µM and 5 µM for FimC and FimI\(_{\text{ox}}^U\).

### 4.8.2. Oxidative folding of FimI\(_{\text{red}}^U\) by DsbA\(_{\text{ox}}^U\)

To see whether the donor strand of FimI might have an effect on oxidation of FimI, we measured DsbA-dependent oxidation of FimI\(_{\text{ox}}^U\), a FimI variant lacking its own donor strand. Based on our previous result that only oxidized FimI\(_{\text{ox}}^U\) can be recognized by FimC, we first determined the presence of free thiols in FimI\(_{\text{red}}^U\) by Ellman’s assay (Ellman 1959). Figure 20A shows oxidation kinetics of FimI\(_{\text{red}}^U\) measured by monitoring the tryptophan fluorescence increase due to the reduction of DsbA\(_{\text{ox}}^U\) (Wunderlich et al. 1993) and oxidation of FimI\(_{\text{red}}^U\). The second-order rate constant yielded for the oxidation of FimI\(_{\text{red}}^U\) (0.75 µM) by DsbA\(_{\text{ox}}^U\) (0.75 µM) at equimolar concentrations was \(2.62 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) (Figure 20A, grey trace). Under the pseudo-first-order conditions FimI\(_{\text{red}}^U\) (0.75 µM) and DsbA\(_{\text{ox}}^U\) (5 µM), the second-order rate constant of oxidation of FimI\(_{\text{ox}}^U\) was \(2.77 \times 10^5 \text{ M}^{-1} \text{s}^{-1}\) (Figure 20A, black trace). Since oxidation and complex formation, have the same increasing signal change, with two spectroscopic probes FimC and DsbA\(_{\text{ox}}^U\) to preclude strong fluorescence contributions coming from DsbA\(_{\text{ox}}^U\), we used a spectroscopically silent variant of DsbA\(_{\text{ox}}^U\) (DsbA\(_{W76FW126F}\)). DsbA\(_{W76FW126F}\) has a wild-type-like catalytic properties (Hennecke et al. 1997), allowed us selective observation of FimC-mediated FimI\(_{\text{ox}}^U\) folding and yielded the second-order rate
Results

constant of $3.77 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ shown in Figure 20B. Next, we measured the kinetics of coupled oxidation of Fiml$^{t}_{\text{red}}$ by DsbA$_{\text{ox}}$ and subsequent binding/folding of Fiml$^{t}_{\text{ox}}$ on the surface of FimC. Since the rate constants for oxidation of Fiml$^{t}_{\text{red}}$ by DsbA$_{\text{ox}}$ ($2.62 \times 10^5 \text{M}^{-1}\text{s}^{-1}$) and complex formation between Fiml$^{t}_{\text{ox}}$ and FimC ($3.4 \times 10^5 \text{M}^{-1}\text{s}^{-1}$, see Figure 19A, 19B and 20B) are very similar, we varied the concentration of FimC to better distinguish the kinetics of the combined oxidation and binding/folding reactions. The reaction with initial concentrations of $5 \text{µM}$ DsbA$_{\text{ox}}$, $5 \text{µM}$ FimC and $0.75 \text{µM}$ Fiml$^{t}_{\text{red}}$ is shown in Figure 20C, whilst the same reaction but with $10 \text{µM}$ FimC is shown in Figure 20D. The reaction of coupled oxidation and folding of Fiml$^{t}_{\text{red}}$ by DsbA$_{\text{ox}}$ and FimC shows the same signal change characterized by increase in tryptophan fluorescence change. The fluorescence change was analysed according to two consecutive, irreversible steps A$\rightarrow$B$\rightarrow$C (see equation in chapter 7.13). The experimentally obtained pseudo-first-order rate constants ($k_{\text{1app}}$, $k_{\text{2app}}$) obtained from the coupled reaction (Figure 20C, 20D) and Table 4 of Fiml oxidation and binding to FimC were consistent within experimental error to those obtained for the individual reactions (Figure 20A, 20B). Based on our kinetics results, disulfide-bond formation in Fiml$_t$ prior to complex formation with FimC is necessary. This also illustrates the mechanism, in which oxidation of Fiml must precede complex formation of Fiml with FimC. The same mechanism has been shown for the FimH, FimA, FimG (Crespo et al. 2012) and now also for termination subunit Fiml.

20A

\[
\text{Fiml}^{t}_{\text{red}} + \text{DsbA}_{\text{ox}} \rightarrow \text{Fiml}^{t}_{\text{ox}} + \text{DsbA}_{\text{red}}
\]

20B

\[
\text{Fiml}^{t}_{\text{red}} + \text{DsbA}_{\text{ox}} + \text{FimC} \rightarrow \text{Fiml}^{t}_{\text{ox}} + \text{FimC} + \text{Fiml}_{\text{N}}
\]
20C

Figure 20: Stopped-flow tryptophan fluorescence kinetics of DsbA\textsuperscript{ox}-dependent oxidation of FimI\textsubscript{t}\textsuperscript{red} and FimC-catalyzed folding of FimI\textsubscript{t}\textsuperscript{ox} at pH 8.0, 25 °C. 20A: The kinetics of tryptophan fluorescence change at equimolar concentrations of FimI\textsubscript{t}\textsuperscript{red} (0.75 µM) and DsbA\textsuperscript{ox} (0.75 µM) (grey dots). The black line corresponds to a fit according to a bimolecular reaction (A+B→C), where [A]=[B]. The kinetics of FimI\textsubscript{t}\textsuperscript{red} oxidation under the pseudo-first-order conditions FimI\textsubscript{t}\textsuperscript{red} (0.75 µM) and DsbA\textsuperscript{ox} (5 µM) (black dots). The green solid line corresponds to a fit according to first-order kinetics. 20B: The kinetics of the combined oxidation with spectroscopically silent variant of DsbA\textsubscript{yy}\textsuperscript{ox} (5 µM), FimI\textsubscript{t}\textsuperscript{red} (0.75 µM) and folding by FimC (5 µM). The disulfide bond formation is spectroscopically silent step and subsequent formation of FimC-FimI\textsubscript{N} complex is evident by a lag phase (reaction between 0s -2.5s). 20C: The kinetics of the combined oxidation and folding were evaluated according to the mechanism of two consecutive, irreversible reactions: A→B→C. The DsbA\textsuperscript{ox} and FimC, both added at initial concentrations of 5 µM, were in > 5-fold molar excess over FimI\textsubscript{t}\textsuperscript{red}. Using a fit according to A→B→C mechanism, 2 pseudo-first-order rate constants (k\textsubscript{1} and k\textsubscript{2}) were obtained (see equation, chapter 7.13). The fit shown in green line was performed using Berkeley Madonna 8.3 program (Macey & Oster). 20D: The same reaction as in 20C, however FimC concentration was increased to 10 µM to see difference between oxidation and complex formation and analysed using the mechanism (A→B→C) (see equation, chapter 7.13). All kinetics rate constants are summarized in Table 4 and all figures were generated in OriginPro9 program.
<table>
<thead>
<tr>
<th>Fig</th>
<th>Conditions</th>
<th>mechanism</th>
<th>$k_1$ (Oxidation) ($M^{-1}s^{-1}$)</th>
<th>$k_2$ (Binding) ($M^{-1}s^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>20A</td>
<td><strong>Equimolar concentration</strong>&lt;br&gt;0.75 µM FimI$^U_{\text{red}}$ + 0.75 µM DsbA$^\text{ox}$&lt;br&gt;<strong>Pseudo-first-order</strong>&lt;br&gt;0.75 µM FimI$^U_{\text{red}}$ + 5 µM DsbA$^\text{ox}$</td>
<td>$I^U_{\text{red}} \overset{k_1}{\underset{k_2}{\rightarrow}} I^U_{\text{ox}}$&lt;br&gt;$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^U_{\text{ox}}$</td>
<td>(2.77 ± 0.03) x $10^5$&lt;br&gt;(2.62 ± 0.02) x $10^5$</td>
<td>-&lt;br&gt;-</td>
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<tr>
<td>20B</td>
<td>0.75 µM FimI$^U_{\text{red}}$ + 5 µM DsbA$^\text{yy}$&lt;br&gt; + 5 µM FimC</td>
<td>$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^U_{\text{ox}}$&lt;br&gt;$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^N_{\text{C}}$</td>
<td>Lag phase</td>
<td>(3.77 ± 0.06) x $10^5$</td>
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<td>20C</td>
<td>0.75 µM FimI$^U_{\text{red}}$ + 5 µM DsbA$^\text{ox}$&lt;br&gt; + 5 µM FimC</td>
<td>$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^U_{\text{ox}}$&lt;br&gt;$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^N_{\text{C}}$&lt;br&gt;$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^N_{\text{C}}$</td>
<td>(2.59 ± 0.05) x $10^5$&lt;br&gt;(2.62 x 10$^5$&lt;br&gt;(3.36 ± 0.01) x $10^5$</td>
<td>3.34 x$10^5$&lt;br&gt;(fixed rate)</td>
</tr>
<tr>
<td>20D</td>
<td>0.75 µM FimI$^U_{\text{red}}$ + 5 µM DsbA$^\text{ox}$&lt;br&gt; + 10 µM FimC</td>
<td>$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^U_{\text{ox}}$&lt;br&gt;$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^N_{\text{C}}$&lt;br&gt;$I^U_{\text{red}} \overset{k_1}{\rightarrow} I^N_{\text{C}}$</td>
<td>2.6 x $10^5$&lt;br&gt;2.6 x $10^5$&lt;br&gt;(fixed rate)</td>
<td>(fixed rate)</td>
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Table 4: Summary of all rate constants of DsbA-dependent oxidation of FimI, and FimC-catalyzed folding of FimI. All rate constants were obtained by stopped-flow tryptophan fluorescence kinetics at pH 8.0, 25 °C. All reactions were measured under the pseudo-first-order conditions, where FimC and DsbA$^\text{ox}$<br>DsbA$^\text{yy}$ were always present in > 5-fold molar excess over FimI.
5. RESULTS II: Protein structure determination by X-ray crystallography

Statement of contribution: Data from both crystal structures, FimC-FimIₜ and FimC-FimIₜ-FimD₅TD(1-125) were processed and solved by Dr. Martin Schärer. Structural analysis was performed with help of Dr. Guido Capitani and Dr. Martin Schärer.

5.1. X-ray structure of FimC-FimIₜ binary complex

To get further insight into the interactions between the chaperone FimC and the termination subunit FimI and into the molecular mechanism of pilus growth termination by FimI, we solved the X-ray structure of the FimC-FimIₜ complex. At high concentration full-length FimI has a tendency to aggregate. Therefore we used a N-terminally truncated FimI variant (FimIΔ1-20) lacking residues 1-20. The N-terminal extension of free FimI is unstructured and not part of the immunoglobulin-like fold (Choudhury et al. 1999, Puorger et al. 2008). The complex was purified to homogeneity and crystallized in 4.5 M sodium formate and 1 M sodium cacodylate at pH 6.5. The obtained crystals were predominately growing in clumps (Figure 21A) with diameters up to 400 µM and diffracted to a maximum resolution of 1.75 Å (Figure 21B).

Figure 21: Crystal clump of FimC-FimIₜ complex and diffraction pattern of the best diffracting crystal. 21A: Hexagonal crystals can be grown reproducibly and tend to grow together in clumps after 3 days at
4 °C. Clumps of crystals reached sizes up to 400 µM. Scale bar: 100 µM. 21B: Diffraction pattern of the best diffracting FimC-FimI crystal. The diffraction pattern was recorded with a Pilatus 2 M pixel detector (exposure time: 0.1 s, angle increment 0.1 deg) at beamline X06DA of the Swiss Light Source (PSI Villigen). Diffraction extends to 1.75 Å resolution.

All FimC main chain atoms were resolved except for the region corresponding to a flexible loop connecting the donor strand of FimC (residues 92-98 were missing from the electron density). Apparently the region between strands D and E (residues 95-99) of FimI is not visible in the electron density due to inherent flexibility. The solvent content of the FimI-FimC crystals is 76.9 %, which corresponds to a $V_M$ of 5.3 Å$^3$/Da. $V_M$ values were calculated based on the unit cell dimensions and the molecular weight of the protein complex in the asymmetric unit (Matthews 1968). The most commonly observed values of $V_M$ are around 2.15 Å$^3$/Da in a range of 1.6-3.5 Å$^3$/Da (Matthews 1968). Higher $V_M$ values correspond to progressively looser packing of the molecules, but still with sufficient intermolecular contacts to stabilize the crystal lattice (Matthews 1968). The total solvent-accessible surface area that is buried between FimI and FimC is roughly 1775 Å$^2$ as calculated by EPPIC (Duarte et al. 2012). In the FimC-FimI structure, FimC donates its $G_1$ β strand to complete the immunoglobulin-like fold of the FimI subunit via a mechanism called DSC (Choudhury et al. 1999, Sauer et al. 1999, Zavialov et al. 2003). The donated $G_1$ strand of FimC runs parallel to the F strand of FimI and is inserted into a deep hydrophobic groove on the surface of FimI (Figure 22D, hydrophobic residues in red). The conserved N- and C-terminal regions of pilus subunits are shown to participate in the quaternary interactions of the mature pilus following their uncapping by the chaperone. Similarly to what was shown for FimC-FimA$_t$ structure (Crespo et al. 2012), FimC interacts via 18 hydrogen bonds with FimI$_t$ residues 149-160, located at the C-terminus of FimI$_t$. Figure 22C shows a close-up view of the inserted G-strand of FimC (pale yellow) with its conserved motif of solvent-exposed hydrophobic residues at positions Asn101(P4), Leu103(P3), Leu105(P2), and Ile107(P1) in the G-strand of FimC (Hung et al. 1996). The conserved disulfide bond between Cys24 (strand A1, chain B) and Cys64 (strand B, chain B) shown in Figure 22E (in yellow) is in a solvent-inaccessible environment. A close-up view of the disulfide bond, exhibiting a distance of 4.9 Å between the C-alpha carbons of its cysteine residues, is given in Figure 22E (in cyan). A distance of 2 Å is between sulfhydryls-gama of cysteines.
Figure 22: Crystal structure of the FimC-FimI, in ribbon and surface representation. 22A: Ribbon representation of the FimC-FimI structure. FimI: grey and FimC: pale yellow. FimI possesses a typical
pilus subunit fold, with 6 ß strands (named A-F) and 2 short α-helices (Choudhury et al. 1999). The G-strand of FimC (yellow) is inserted in a hydrophobic groove of FimI between strands A1, A2 and F (shown in grey). 22B: Surface representation of FimC-FimI structure. 22C: Close-up view of the interactions between the G-strand of FimC and strands A1 and A2 of FimI. 22D: Surface representation of FimI color-coded by hydrophobicity and showing the hydrophobic groove in which G strand of FimC is inserted (color code: pale yellow, blue and red for carbon, nitrogen and oxygen atoms, respectively). Red denotes hydrophobic residues, grey hydrophilic ones. 22E: Topology diagram and close-up view (right panel) of the disulfide bond between two conserved cysteines (Cys-24 of strand A1) and (Cys-64 of strand B). The distance of 4.9 Å between the Cys C-alpha atoms is denoted by a blue dashed line. All Figures were prepared using PyMOL (Schrodinger 2010).

5.2. X-ray structure of ternary complex FimC-FimI-FimDNTD(1-125)

There are still many open questions about the termination of type 1 pili. Here we tested whether the termination mechanism might hinge on a failure by the termination subunit-chaperone complex to bind the N-terminal domain of FimD. As reported for the FimC-FimHp-FimDNTD(1-125) structure, both the N-terminal segment (“tail” residues 1-24) and the globular core domain (“body” residues 25-125) of FimDNTD(1-125) contribute to chaperone-subunit recognition and recruitment (Nishiyama et al. 2003, Ng et al. 2004, Nishiyama et al. 2005).

To get more information about the termination mechanism by FimI, we tested the binding ability of the FimC-FimI complex to FimDNTD(1-125) depicted by the analytical size-exclusion chromatography in Figure 23A (see chapter 7.11). We used an N-terminally truncated variant of FimI without a disordered segment (1-20 residues) that might impair crystallization. For the N-terminal domain of FimD, we used a variant (1-125) containing the N-terminal “tail” residues 1-24 and the structural core residues 25-125. Analytical SEC confirmed binding of the N-terminal domain of FimD to the FimC-FimI complex (see Figure 23A, peak eluted at 11.5 ml). The calculated molecular masses of monomeric FimDNTD(1-125) and of the FimC-FimI and FimC-FimI-FimDNTD(1-125) complexes are 13.66 kDa, 38.55 kDa, 54.34 kDa respectively. To unravel the structural basis of the interaction between FimC-FimI and FimDNTD in more detail, we used crystallography and obtained a crystal form of the FimC-FimI-FimDNTD(1-125) ternary complex in space group P2₁2₁2₁, which is the most frequently seen in protein crystals (Wukovitz and Yeates 1995) (see Figure 23B and Crystallographic Table 5). The high resolution electron density map showed that FimDNTD(1-125) lacks electron density for residue positions 10-15. Density for these residues is also missing in structures FimC-FimHp-
FimD\textsubscript{NTD(1-125)} (CHD) (Nishiyama et al. 2005) and in FimC-FimF-FimD\textsubscript{NTD(1-125)} (CFD) (Eidam et al. 2008). A remarkable difference is found in FimC region 91-101, which is more ordered in FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD(1-125)} (CID) due to additional interactions brought upon by the binding of FimC-FimI\textsubscript{t} to FimD\textsubscript{NTD(1-125)}. This region is usually lacking electron density as seen in the FimC-FimA\textsubscript{t} (Crespo et al. 2012) and FimC-FimI\textsubscript{t} structures probably due to the inherent flexibility of its loop. Also, in FimI\textsubscript{t}, residues 119-121 (3aa) are lacking electron density. The FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD(1-125)} crystals exhibit a solvent content of 47.0% and a V\textsubscript{M} of 2.32 Å\textsuperscript{3}/Da. The crystal lattice of FimC-FimI\textsubscript{t} is thus more loosely packed than that of FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD(1-125)}.

Furthermore, in the FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD(1-125)} structure, the FimD\textsubscript{NTD(1-125)} core forms no direct contacts with the FimI\textsubscript{t} pilin domain. FimC interacts via its F-strand (residues 1–116) with both the pilin domain of FimI\textsubscript{t}, through donor strand complementation, and with the globular core of FimD\textsubscript{NTD(1-125)}, that is residues 25-125.

Figure 23: X-ray structure of ternary complex FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD(1-125)}. 23A: Analytical gel filtration shows the formation of the FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD(1-125)} ternary complex. All proteins were present at 15 µM concentration in 20 mM Tris/HCl pH 8.0 at 25 °C. Elution volumes of standard proteins with the corresponding molecular masses are indicated above the plot. Molecular weights of proteins are indicated in Table 8. 23B: Side-view of the ribbon representation of the FimC-FimI\textsubscript{t} interface with FimD\textsubscript{NTD(1-125)} at 1.75 Å resolution. FimC is depicted in yellow, FimI\textsubscript{t} in grey, the N-terminal domain of FimD in green. Figure was prepared with PyMOL (Schrodinger 2010).
<table>
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<th>FimC-FimI&lt;sub&gt;t&lt;/sub&gt;</th>
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**Refinement**

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**Table 5: Crystallographic table.**

Data were collected from single crystals.

i) Statistics for outer resolution shell given in parantheses

ii) Ramachandran analysis carried out with PHENIX
5.3. Detailed characterization of FimD<sub>NTD(1-25)</sub>–FimC interface

An important feature of the N-terminal FimD<sub>NTD</sub> tail is the crowding of three aromatic residues, Phe4, Phe8 and Phe22, which contribute hydrophobic contacts to the interface between FimD<sub>NTD(1-125)</sub> and the FimC–FimI<sub>t</sub> complex. Based on data from FimC–FimH<sub>p</sub>–FimD<sub>NTD(1-125)</sub> (Nishiyama et al. 2005) and FimC–FimF<sub>t</sub>–FimD<sub>NTD(1-125)</sub> (Eidam et al. 2008), the hydrophobic interactions are likely to be key to the stability of the FimC–FimI<sub>t</sub>–FimD<sub>NTD(1-125)</sub> structure and to the recognition of FimC–FimI<sub>t</sub> by FimD<sub>NTD(1-125)</sub>. Inspection of the protein interface shared by residues from the 1-25 tail of FimD and by FimC reveal that Phe4, Phe8, Phe22; pale green interact with conserved FimC “hot spot” residues (Leu32, Ile90; pale-yellow). These interactions were found in all three available ternary structures CID, CHD and CFD shown in Figure 24A, B, C, respectively. The mutations of key residues Leu32 and Ile90 of FimC abolished pilus formation in vivo (Nishiyama et al. 2005).

![Figure 24: Stereo representation of the interfaces between FimC and FimD<sub>NTD</sub> in different complexes. 24A: Close-up view of the tail interface of FimD<sub>NTD</sub> and FimC in the CID structure. The molecular surface of FimC is shown in pale yellow, that of FimI<sub>t</sub> in grey. Detailed depiction of hydrophobic residues from FimD<sub>NTD</sub> (Phe4, Phe8 and Phe22), shown in green as sticks, interacting with residues of FimC Leu32 and Ile90. 24B: Close-up view of the tail interface of FimD<sub>NTD</sub> and FimC in the CHD structure. The molecular surface of FimH<sub>p</sub> is shown in cyan. 24C: Close-up view of the tail interface of FimD<sub>NTD</sub> and FimC in CFD structure. The molecular surface of FimF<sub>t</sub> is shown in brown. All figures were prepared with PyMOL (Schroedinger 2010) and interfaces were characterized using EPPIC (Duarte et al. 2012)]
5.4. Detailed characterization of $\text{FimD}_{\text{NTD}(1-25)}$–$\text{FimI}_t$ interface

According to hydrogen bond (HB) plus calculation there is no single direct contact of $\text{FimI}_t$ with $\text{FimD}_{\text{NTD}(1-25)}$ interacting via hydrogen bonds. It might be due to non-correct orientation in hydrogen bonds in $\text{FimI}_t$. However $\text{FimI}_t$ clearly interacts with $\text{FimD}_{\text{NTD}(1-25)}$ through Vander Wals interactions through 7 residues (Val79, Ser89, Val90, Gly91, Gln152, Ala 153 and Trp 154). The most interesting is interaction with Gln152 and Asn5 of FimD of about 3.8 Å (Figure 25), which can be considered as a “weaker hot spot” (84% buried). The interface between $\text{FimD}_{\text{NTD}}$ and $\text{FimI}_t$ is slightly large at 217 Å$^2$ compared to $\text{FimD}_{\text{NTD}}$–$\text{FimF}_t$ (126 Å$^2$), and similar to than $\text{FimD}_{\text{NTD}}$–$\text{FimH}_p$ interface (213 Å$^2$). The main interface in CID is between $\text{FimD}_{\text{NTD}}$ and FimC at (1006 Å$^2$), what is comparable to CHD at (1093 Å$^2$) and in CFD at (979 Å$^2$).

Figure 25: Stereo representations of the interfaces between tail of FimD and $\text{FimI}_t$ subunit. Close-up view of the interactions between $\text{FimI}_t$ (grey) and $\text{FimD}_{\text{NTD}}$ (pale green), both partners are shown in cartoon and stick representation. Figure was prepared with PyMOL (Schrodinger 2010) and interfaces were characterized using EPPIC (Duarte et al. 2012).

Another notable features are the conformational changes in $\text{FimI}_t$ that occur upon binding of the FimC-$\text{FimI}_t$ complex to $\text{FimD}_{\text{NTD}(1-125)}$, shown in Figure 26. There both $\text{FimI}_t$ from a free FimC-$\text{FimI}_t$ complex and from the $\text{FimD}_{\text{NTD}(1-125)}$-bound FimC-$\text{FimI}_t$ complex appear in grey. The regions undergoing conformational changes in the latter complex appear in cyan. The binary complex of FimC-$\text{FimI}_t$ was superimposed onto the FimC-$\text{FimI}_t$-$\text{FimD}_{\text{NTD}(1-125)}$ ternary complex, of which only $\text{FimI}_t$ and the 1-25 region of $\text{FimD}_{\text{NTD}(1-125)}$ are displayed in Figure 26.
The conformational changes mainly involve residues located on the F-strand of FimI<sub>t</sub>. The upper panel of Figure 26 describes the detailed situation, in which the side chain of Gln152 rotates by roughly 90° about the vertical axis: its carboxamide oxygen interacts with the guanidinium moiety of Arg7 from FimD<sub>NTD(1-125)</sub> (3 Å distance). Not only does Gln152 of FimI<sub>t</sub> undergo a conformational change, but also Arg7 of FimD<sub>NTD(1-125)</sub> is shifted compared to the FimC-FimH<sub>Trp</sub>-FimD<sub>NTD(1-125)</sub> structure (Nishiyama et al. 2005). Trp154 and Phe155 get ordered more than 90° position change (Figure 26, right lower panel). The interacting residues of FimI<sub>t</sub> are mainly in its C-terminal part, which contains many conserved residues. Among those, Phe155, Ser156 and Trp154 undergo conformational changes upon binding to FimD<sub>NTD(1-125)</sub>. The conformational changes are clearly not caused by crystal packing effects.

Figure 26: Conformational changes in FimI<sub>t</sub> upon binding FimC-FimI<sub>t</sub> to FimD<sub>NTD(1-125)</sub>. Superposition of FimI<sub>t</sub> from a FimC-FimI<sub>t</sub> binary complex (F-strand depicted in grey) onto FimI<sub>t</sub> from a FimC-FimI<sub>t</sub>-FimD<sub>NTD</sub> ternary complex (F-strand depicted in cyan). The conformational changes undergone by Ser156, Phe155, Trp154 and Gln152, located on the F-strand of FimI<sub>t</sub>, upon binding to FimD<sub>NTD(1-125)</sub> are shown. The zoomed-in view in the upper right panel shows the detailed interaction of Gln152-OE19 of FimI<sub>t</sub> with Arg7-NE of FimD<sub>NTD(1-125)</sub>. The zoomed-in view in the lower right panel shows the conformational changes of conserved residues from the C-terminal part of FimI<sub>t</sub>. The pink arrows indicate changes in residues.
Ser156, Phe155 and Trp154. Residues from the unliganded FimC-FimI\textsubscript{t} complex appear in grey, those from FimC-FimI\textsubscript{t} bound to FimD\textsubscript{NTD(1-125)} appear in cyan.

5.5. Proposed catalytic mechanism of termination by FimI

The N-terminal extension (Nte) or donor strand of pilus subunits contains a highly conserved array of alternating hydrophobic residues, termed ‘P2–P5 residues’ (see Figure 27, residues in red) (Soto et al. 1998, Sauer et al. 1999). Each subunit donates its Nte to complete the immunoglobulin fold of the preceding subunit, inserting its P2–P5 residues into the corresponding P2–P5 binding pockets in the groove of that subunit (Jacob-Dubuisson et al. 1994, Sauer et al. 2002, Zavialov et al. 2003, Verger et al. 2006). The ability of the incoming subunit to initiate a donor-strand exchange reaction with the previously assembled subunit is crucially dependent on a defined binding site, called P5 (Remaut et al. 2006). The P5 site allows for displacement of the chaperone donor-strand through a concerted $\beta$ strand displacement mechanism, called a step-wise zip-in-zip-out mechanism (Remaut et al. 2006, Verger et al. 2006, Rose et al. 2008, Verger et al. 2008). It is also described as a critical point for termination in the Pap system, where it was shown that the P5 pocket of the PapH termination subunit is closed and therefore cannot accept the N-terminal extension of an incoming pilus subunit (Verger et al. 2006). In both systems, Pap and Fim, the P5 pocket is not occupied by side chains of the G strand of the FimC chaperone. In the case of SafA (pilin subunit in Salmonella enterica), the P5 region was completely disordered (Remaut et al. 2006). Here, we demonstrate the existence of two states of the P5 pocket in the Fim system, open and closed. Interestingly the P5 pocket of FimI is described by Ile, while in other pilus subunits is shown Val (see Figure 27). The open conformation of the P5 pocket is shown in the FimI\textsubscript{t} subunit (extracted from FimC-FimI\textsubscript{t} structure) in Figure 28A. The closed P5 pocket and not accessible for an incoming donor strand is shown in FimI\textsubscript{t} (extracted from FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD(1-125)}) structure in Figure 28B. The proposed closure mechanism of P5 can be followed by comparing the region of P5 pocket in the three available structures of FimI\textsubscript{t}, where Figure 28C shows FimI\textsubscript{t} from a binary complex FimC-FimI\textsubscript{t} (open), Figure 28D FimI\textsubscript{t} from a ternary complex FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD(1-125)} (closed). Figure 28E shows FimI\textsubscript{wt} from a ternary complex FimC-FimI\textsubscript{t}-FimA\textsubscript{t} (closed) (Ignatov 2009). In ternary structure of FimC-FimI\textsubscript{t}-
FimD<sub>NTD(1-125)</sub>, the P5 pocket gets closed by a kink, which causes conformational rearrangements in donor strand of FimC.

Figure 27: The structural alignment of type 1 pilus subunit's sequences. The program Multiple Align Show (www.bioinformatics.org/sms) was used to structurally align FimH<sub>p</sub>, FimG, FimF, FimA and Fiml sequences of <i>E. coli</i> K12 W3110. The degree of residue conservation between 5 subunits is denoted by black for highly conserved (identical) and blue for similar amino acid residues. The N-terminal donor strand sequences with highlighted binding pockets (in red) of the individual subunits were manually added.
Figure 28: Existence of two states of the P5 pocket (open and closed) in FimI subunits. 28A: The surface representation of FimI (in grey) extracted from a binary complex FimC-FimI. The G-strand of FimC is colored in violet. The P5 pocket is marked by a red circle and shows an open conformation of P5. 28B: The surface representation of FimI (in grey) extracted from a ternary complex FimC-FimI-FimD_{NTD(1-125)}. The G-strand of FimC is colored in cyan. The P5 pocket is indicated by a red circle and shows a closed conformation. 28C: Ribbon representation of FimI-dsFimC, same as shown in 28A. 28D: Ribbon
representation of FimI-dsFimC, same as shown in 28B. The red circle indicates a helix formed after binding FimC-FimI to FimD_{NTD(1-125)} as a reason for conformational changes in P5 pocket. **28E:** Ribbon representation of FimI-dsFimC (yellow) extracted from FimC-FimI-FimA complex (Ignatov 2009). All pictures were prepared with PyMOL (Schrodinger 2010).

A similar closing mechanism was found by Verger et al. in the *pap* system (Verger et al. 2006, Verger et al. 2008). In the termination subunit PapH, Thr52 obstructs the P5 pocket of PapH because it is forced into the observed conformation by a kink caused by Pro 53 (Verger et al. 2006).

To investigate possible peculiarities in the interface of FimD_{NTD(1-125)} with FimC-FimI, we superimposed the FimC-FimI-FimD_{NTD(1-125)} structure onto that of FimC-FimH_{p}-FimD_{NTD(1-125)} (PDB code 1ze3). The FimC-FimI-FimD_{NTD(1-125)} structure turns out to be in very close agreement with that of FimC-FimI-FimD_{NTD(1-125)} with a r.m.s.d. of 1.029 Å for 2110 common C\(^\alpha\) atoms. Moreover, the structure of FimC-FimF_{p}-FimD_{NTD(1-125)} (pdb code 3BWW) did superimpose onto that of FimC-FimI-FimD_{NTD(1-125)} with a r.m.s.d. of 0.990 Å for 2127 common C\(^\alpha\) atoms. The total buried interface area between FimD_{NTD(1-125)} and FimC is 980 Å\(^2\) in FimC-FimF_{p}-FimD_{NTD(1-125)} (Eidam et al. 2008), 1090 Å\(^2\) for FimC-FimH_{p}-FimD_{NTD(1-125)} and 1010 Å\(^2\) in FimC-FimI-FimD_{NTD(1-125)}. All area values for the interfaces between FimD_{NTD(1-125)} and the chaperone-subunit complexes were obtained with the EPPIC server (Duarte et al. 2012), which contains precalculated interface classification results for all structures in the PDB (Baskaran et al. 2014). Moreover, an open P5 pocket was found in both structures CFD (Figure 29A) and CHD (Figure 29B), while in CID P5 pocket is closed (Figure 29C). This also demonstrates the function of FimI as a termination subunit, since it cannot undergo DSE reaction due to an obstructed P5 pocket. Superimposition of N-terminal tail of CID, CFD and CHD showed that there is no difference in orientation of the FimD_{NTD} in all structures (Figure 29D).
Figure 29: Close-up view into the P5 pocket in different ternary complexes. 29A: The surface representation of FimF, subunit (brown) and ribbon representation of FimC (pale-yellow) and FimD<sub>NTD</sub> (green). The P5 pocket is marked by a red circle and shows an open conformation of P5. 29B: The surface representation of FimH<sub>p</sub> subunit (cyan) and ribbon representation of FimC (pale-yellow) and FimD<sub>NTD</sub> (green). Same as shown in 29A, P5 pocket is marked by a red circle and shows an open conformation of P5. 29C: The surface representation of FimI, subunit (grey) and ribbon representation of FimC (pale-yellow) and FimD<sub>NTD</sub> (green) with closed P5 pocket in FimI, subunit. 29D: Superimpositions of N-terminal tail of FimD from all three structures CFD, CHD and CID shows no differences in FimD-tail orientation.
When comparing affinity of the chaperone–subunit pairs for binding to FimD<sub>NTD</sub>, the affinity in CFD is lower than in CHD (Nishiyama et al. 2003). Table 6 summarizes all K<sub>d</sub> values for chaperone-subunit complexes (Nishiyama and Glockshuber 2010). Obtained K<sub>d</sub> values of 27.0 ± 3.6 μM and 28.7 ± 3.9 μM for the binding of FimC–FimG<sub>t</sub> and FimC–FimA<sub>t</sub>, respectively, demonstrate that the affinity of these complexes for FimD<sub>NTD</sub> is about 1 order of magnitude weaker than the binding of FimC–FimH and FimC–FimF<sub>t</sub> (0.92 ± 0.08 mM and 6.6 ± 0.6 mM, respectively) (Nishiyama and Glockshuber 2010). According to these values, the affinity for the FimC-FimI<sub>t</sub> complex is very high in comparison with the other pilus subunits. This indicates that the termination machinery of FimI might occur through the blocking FimD<sub>NTD(1-125)</sub> in a FimC-FimI<sub>t</sub> bound conformation. The binding of FimC-FimI<sub>t</sub> complex to CTD domain of FimD has not yet been clearly investigated. We know that since the 170 residue C-terminal domain of FimD (CTD) has poorly understood function and it cannot recognize FimC alone (So and Thanassi 2006, Ford et al. 2010, Geibel et al. 2013). Our preliminary experiments aiming to obtained crystal structure of ternary complex FimC-FimI<sub>t</sub>-FimD<sub>CTD</sub> were unsuccessful probably due to low affinity of CTD to bind FimC-FimI<sub>t</sub> complex (data not shown) or the unability of FimC-FimI<sub>t</sub> to dissociate from NTD domain of FimD once is bound to it.

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<td>ND</td>
<td>3.4 ± 0.2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>T= 4 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>k&lt;sub&gt;on&lt;/sub&gt; (10&lt;sup&gt;6&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>17</td>
<td>5.7</td>
<td>3.6</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>k&lt;sub&gt;off&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>7</td>
<td>197</td>
<td>21</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt; (=k&lt;sub&gt;off&lt;/sub&gt;/k&lt;sub&gt;on&lt;/sub&gt;) (μM)</td>
<td>0.4</td>
<td>34.6</td>
<td>5.8</td>
<td>28.0</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt; (amplitude)&lt;sup&gt;c&lt;/sup&gt; (μM)</td>
<td>0.5 ± 0.01</td>
<td>34.2 ± 6.9</td>
<td>5.9 ± 0.1</td>
<td>25.3 ± 7.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: A summary of Kd values for all pilus subunits. The table was taken from (Nishiyama and Glockshuber 2010) and values for FimC-FimI<sub>t</sub> complex were measured by Olexander Ignatov (Ignatov 2009).
6. DISCUSSION and OUTLOOK

The adhesive organelles are found in the majority of uropathogenic *E.coli* strains (*UPEC*) causing urinary tract infections (UTIs), including cystitis and pyelonephritis (Bien et al. 2012). Type 1 pili are the most widely distributed among UPEC isolates due to the ability of its adhesin domain FimH to bind to mannosylated residues expressed on human bladder epithelial cells (Krogfelt et al. 1990). This study completes the structural inventory of type 1 pili domains and provides further insight into the termination mechanism by which FimI blocks pilus growth.

The question set up at the beginning of this dissertation thesis was to reveal the reasons for the low periplasmic concentrations of FimI in comparison to the main structural subunit FimA. Previous data from our lab predicted that the *in vivo* concentrations of FimI are much lower than those of FimA, and that FimI stops pilus assembly in a stochastic chain termination reaction. Therefore we first tested the hypothesis whether the slow or inefficient FimC-catalyzed folding of FimI compared to FimA is responsible for its low *in vivo* concentration. First, our studies on FimI alone revealed that FimI can be refolded without help of FimC at pH 5.0 with defined secondary and tertiary structure. However, spontaneous refolding of FimI alone is very slow and aggregate-prone, therefore unsuitable for further quantitative measurements. To understand more what is happening with FimI in the periplasm, we measured the stopped-flow tryptophan fluorescence kinetics of oxidation FimI\textsubscript{U red} by DsbA and FimC-catalysed folding of FimI\textsubscript{U ox} what supports the following mechanism: FimI\textsubscript{U red} + DsbA\textsubscript{ox} $\overset{k_1}{\rightarrow}$ FimI\textsubscript{U ox} + FimC $\overset{k_2}{\rightarrow}$ FimC-FimI\textsubscript{N ox}, where fast DsbA-dependent oxidation of FimI\textsubscript{U red} is followed by the slower folding of FimI on the surface of FimC. We could show that FimI interacts with the periplasmic folding catalysts DsbA and FimC in a similar manner as the main subunit FimA, and that DsbA-catalyzed disulfide bond formation is required for FimC-catalyzed folding of FimI. Together with the known *in vivo* concentrations of DsbA and FimC (Crespo et al, 2012), the obtained rates of DsbA-dependent oxidation and FimC-catalyzed folding of FimI predicted that the *in vivo* half-life of FimI folding is even shorter than folding of FimA (0.7 s compared to 1.1 s, respectively).
Additionally, we showed that FimI was oxidized by DsbA about 30 times faster than FimA, while FimC-catalyzed folding of FimI and FimA proceeded equally fast. Based on obtained rate constants, our results exclude inefficient in vivo folding of FimI as the cause of its low in vivo concentrations, suggesting that FimI may be less efficiently translated or more efficiently degraded in vivo relative to FimA. Since FimI is unique in having the longest donor strand among all other structural pili subunits, to elucidate the reasons for the uniqueness of FimI donor strand, we used a truncated variant of FimI (called FimI_t). FimI_t is an N-terminally truncated variant of FimI depleted of its own donor strand. Therefore we measured stopped-flow tryptophan fluorescence kinetics and compared it with kinetics measured for the full length of FimI. While a donor strand of FimI carries out one tryptophan (W4), now FimI_t is contributing to the tryptophan fluorescence signal change by 2 tryptophans. We found out that DsbA-dependent oxidation of FimI_t was 3 times slow than oxidation of full-length FimI. Moreover, the FimC-catalyzed folding of FimI_t was 10 times faster than folding of FimI, what can be explained by sterical interference of ds in FimI with FimC. In summary, effective FimC-catalysed folding of FimI is pointing out to find other reasons responsible for low FimI periplasmic concentrations.

Since we did not see a significant difference in complex formation between FimC-FimI_Nox (4.05 x 10^4 M^{-1}s^{-1}) and FimC-FimA_Nox (4.39 x 10^4 M^{-1}s^{-1}), we looked for other reasons why FimI might be present at lower concentration in the periplasm of E.coli. If different expression levels of the proteins are taken into account, which were determined to be 5 : 10 : 4 : 100 : 28 for FimH, FimG, FimF, FimA and FimI, respectively (Schembri et al. 2002) the answer might be somewhere on translation level. One reason could be lower expression of FimI due to degenerate Shine-Dalgarno sequence of fimI, which can cause weak binding of ribosome to the Shine-Dalgarno sequence of fimI. It was found to be stabilized by only two Watson-Crick hydrogen bonds compared to three for GC base pairs in an optimal sequence. The second reason could be that translation pausing is driven by anti-Shine-Dalgarno sequences of fimI (Li et al. 2012). Bioinformatics analysis using Artemis software (Rutherford et al. 2000) revealed that fimI possesses two motifs corresponding to anti-Shine-Dalgarno sequences and fimI gene carries two such motifs GGC GGC (Gly-Gly) and GGC GGA (Gly-Gly).
Third, bioinformatics analysis also pointed to the existence of a transcription attenuator immediately after \textit{fimA} and presence of rare AGG/AGA and GGG codons in the \textit{fimI} gene. The clusters of AGG/AGA codons can reduce both the quantity and quality of the synthesized protein. In addition, it is likely that an excess of any of these codons, even without clusters, could create translational problems (Kane 1995). These genetic properties could be the reason for low occurrence of FimI. In this work we have tried to confirm this hypothesis, using in \textit{vitro} translation experiment in collaboration with Dr. Erich Michel (Allain group, ETH) and by several site-direct mutagenesis experiments in \textit{fimI} gene tested by Western blot technique. However, we could not demonstrate exact reasons for low periplasmic concentration of FimI (data not shown).

To provide more insight into interactions between FimC-FimI and usher FimD, we solved the structure of the FimC-FimI\textsubscript{t} (FimI\textsubscript{Δ20}) at 1.75 Å and of FimC-FimI\textsubscript{t} bound to the N-terminal substrate recognition domain of FimD (FimC-FimI\textsubscript{t}-FimD\textsubscript{NTD}) at 1.70 Å. The FimC-pilus subunit binding to FimD\textsubscript{NTD} has been shown so far for 2 pilus subunits: FimC–FimF\textsubscript{t}-FimD\textsubscript{NTD} (Eidam et al. 2008) and FimC–FimH\textsubscript{p}-FimD\textsubscript{NTD} (Nishiyama et al. 2005). The low binding affinity of FimC-FimG\textsubscript{t} (K\textsubscript{d}=30.6 µM) and FimC-FimA\textsubscript{t} (K\textsubscript{d}=27 µM) complexes to bind N-terminal domain of FimD was shown by Dworkowski (Dworkowski 2010, Nishiyama and Glockshuber 2010). The solved structures revealed necessary interactions for binding FimI\textsubscript{t} with chaperone FimC, in which ds of FimC inserts into hydrophobic groove of FimI\textsubscript{t}, runs parallel compared to C-terminal F-strand of FimI\textsubscript{t} and completes the otherwise incomplete Ig-like fold of FimI\textsubscript{t}. Similar to what was observed for FimC-FimH, FimC-FimA\textsubscript{t} and FimC-FimF\textsubscript{t}. Then, the inspection of the protein interface shared by residues from the 1-25 tail of FimD (Phe4, Phe8, Phe2) and by FimC revealed interactions with conserved “hot spot” residues of FimC namely, Leu32 and Ile90.

Recently it was shown that PapH from the P Pilus uropathogenic \textit{Escherichia coli} has an obstructed P5 pocket, what causes inability to undergo DSE between PapD and PapH (Verger et al. 2006). The crystallographic structure of PapD-PapH\textsubscript{Nte} (PDB code 2J2Z) shows a completely closed P5 pocket in a termination subunit PapH. When looking on our ternary crystal structure of FimC–FimI\textsubscript{t}-FimD\textsubscript{NTD}, closed P5 pocket in FimI\textsubscript{t} subunit is evident.
However the P5 pocket stayed open in FimI$_t$ subunit in a binary complex of FimC-FimI$_t$. Because an open P5 pocket has been shown for all other pilus subunits (FimA, FimF, FimG, and FimH), this indicates that FimD$_{NTD}$ can specifically induce a conformational transition in FimC-bound FimI$_t$ that closes P5 pocket of FimI$_t$. To reset the assembly machinery for a new incorporation, the incoming chaperone-subunit complex would need to dissociate from FimD$_{NTD}$ and be transferred to the CTD (C-terminal domain) site of FimD (Phan et al. 2011). Our results showed that in Fim system, the termination subunit-chaperone complex FimC-FimI$_t$ binds to FimD$_{NTD}$, however whether the terminator complex is then directly targeted to the Plug domain or CTD domains of FimD remains still unclear. Because the last incorporated chaperone-subunit is known to remain bound on the usher (Saulino et al. 1998, Ng et al. 2004), it might also be that the pilus termination and anchoring occur by the failure of the handover from FimD$_{NTD}$ domain to the CTD domains of FimD, what is also involved in the termination process. The termination machinery of FimI might occur through blocking FimD$_{NTD}$ in a FimC-FimI$_t$ bound conformation. So far, our investigations on minor subunit FimI answered detailed mechanism of FimI in periplasm and showed that FimI is not fundamentally different from the major pilus subunit FimA, unless is bound to NTD of usher FimD.

Moreover, bioinformatics analysis revealed that FimI can be preferentially recognized by a periplasmic serine protease DegP (HtrA). As a protease, DegP was shown to degrade a large range of unfolded or misfolded substrates what was also shown for PapA subunit (Jones et al. 2002). Because PapA is a homologues subunit to FimA, we hypothesized that FimI can act as a substrate and be bound by the one of the PDZ domains (PDZ1 or PDZ2) of DegP. DegP cleaves its substrates preferentially after Ile, Val, Met, Leu, Thr, Ser and Ala residues by employing a hold-and-cut mechanism (Krojer et al. 2008). Detailed look into a structure FimI$_t$ has revealed that in a comparison with other Fim subunits, FimI carries out specific motif STVV (Serine-Threonine-Valine-Valine). The motif is located on helix, residues 65-68 after second β-beta sheet (Figure 30). The most common serine protease in E.coli K12, which possess binding motif to query PDZ domain (Glycine-Leucine-Glycine-Phenylalanine) motif, is DegP. On the basis of the available biochemical and structural information for DegP PDZ domains we believe that this specific feature would be an explanation for low FimI
periplasmic concentrations. Among all pilus subunits this motif has been observed only for FimI subunit. To test what is happening with FimI after entering the periplasm, whether it is rapidly degraded by DegP it needs to be further investigated.

Figure 30: Close-up view into STVV motif of FimI, subunit necessary for DegP recognition. In a right panel, small cartoon figure shows the mechanism of recognition of unfolded reduced FimI by DegP.
7. MATERIALS and METHODS

7.1. MATERIALS: Chemicals

Bacterial growth-media: Bacto™ Yeast extract, Bacto™ Tryptone (Pancreatic Digest of Casein) and Agar were supplied by BD Becton, Dickinson and Company (Franklin Lakes, USA). For expression of proteins in the cytoplasm the *E.coli* strain BL21 (DE3), BL21 (DE3) pLys, BL21 (DE3) Tuner™ from Novagen (Germany) were used (see Table 7) (Studier and Moffatt 1986, Pan and Malcolm 2000).

Chemicals were purchased from Merck (USA), Sigma-Aldrich (St. Louis, USA), Applichem (Germany), Roth (Germany). Guanidium chlorid (GdmCl) used in this thesis was from NiGu Chemie GmbH (Waldkraiburg, Germany). For spectroscopical measurements „AA-Grade“ Guanidium chloride was used. Ethylenediaminetetraacetic acid (EDTA), Tetramethylethylenediamine (TEMED), Glycerol and Dithiothreithol (DTT) were obtained from Applichem (Germany). Polymyxin B sulfate was purchased from Sigma-Aldrich (St. Louis, USA). FimA antibodies were produced by ProteoGenix SAS (France) and DNA oligonucleotides were obtained from Microsynth (Switzerland).

7.2. Chromatographic columns and column materials

Ion exchange columns: Resource™ S 1 ml and S 6 ml and Resource™Q 1 ml and Q 6 ml (GE Healthcare, USA), SEC preparative columns: Superdex 75 26/60 and Superdex 200 26/60, analytical SEC columns: Superdex 75 10/300 GL (GE Healthcare, USA). Self-packed 14 ml HisTrap™ IMAC column, QA52, phenyl sepharose and self-packed 60 ml Desalting Sephadex G-25 column were used. Ni²⁺-NTA medium for histidine selective metal affinity-chromatography was from Qiagen (Hilden, Germany). Medium for anion exchange resin QA52 was purchased from Whatman (Kent, UK) and for Sephadex G-25 from (GE Healthcare, USA).
7.3. *E.coli* strains used in this study

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21 (DE3)</td>
<td>F–ompT gal dcm lon hsdS8 (rB– mB–) λ(DE3) : (lacI, lacUV5-T7 gene 1, ind1, sam7, nin5)</td>
<td>(Studier and Moffatt 1986)</td>
</tr>
<tr>
<td>BL21 (DE3) pLys</td>
<td>F–ompT gal dcm lon hsdS8 (rB– mB–) pLys (CamR) λ(DE3) : (lacI, lacUV5-T7 gene 1, ind1, sam7, nin5)</td>
<td>(Pan and Malcolm 2000)</td>
</tr>
<tr>
<td>BL21 (DE3) Tuner</td>
<td>F–ompT gal dcm lon hsdS8 (rB– mB–) gal dcm lacY1 λ(DE3) : (lacI, lacUV5-T7 gene 1, ind1, sam7, nin5)</td>
<td>(Novagen, Germany)</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>endA1 gyrA96 thi-1 recA1 relA1 hsdR17(rk–, mk+) supE44 λ lac T7[proAB lacI Delta(lacZ)M15 Tn10(tet)]]</td>
<td>(Bullock et al. 1987)</td>
</tr>
<tr>
<td>HM125</td>
<td>F–ΔlacX74 galE galK thi rpsL(strA) ΔPhoA(PvuII) degP eda rpoH15</td>
<td>(Meerman and Georgiou 1994)</td>
</tr>
</tbody>
</table>

Table 7: A summary of all *E.coli* strains used in this study.

7.4. Proteins used in this study and their properties

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mr(Da)</th>
<th>#aa</th>
<th>pI</th>
<th>$\varepsilon_{280}$ (M$^{-1}$ cm$^{-1}$)</th>
<th>Used plasmid</th>
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<tr>
<td>FimI$_{ox}$</td>
<td>17169</td>
<td>160</td>
<td>8.02</td>
<td>21 100</td>
<td>pET11a/pfimIcyt</td>
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<tr>
<td>FimI$_{ct}$</td>
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<td>140</td>
<td>7.12</td>
<td>15 600</td>
<td>pET11a/pfimI</td>
</tr>
<tr>
<td>FimC</td>
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<td>211</td>
<td>10.1</td>
<td>22 860</td>
<td>pET11a/pfimC27</td>
</tr>
<tr>
<td>FimC$_{His}$</td>
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<td>211</td>
<td>10.0</td>
<td>14 250</td>
<td>pET11a/pfimC27</td>
</tr>
<tr>
<td>FimA$_{ox}$</td>
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<td>159</td>
<td>4.5</td>
<td>3 110</td>
<td>pET11a/pfimCW36YW84Yhis-</td>
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<tr>
<td>DsbA$_{ox}$</td>
<td>21132</td>
<td>189</td>
<td>5.42</td>
<td>23 050</td>
<td>pET11a/pfimAwt-cyt</td>
</tr>
<tr>
<td>DsbA$_{W76FW126F}$</td>
<td>21054</td>
<td>189</td>
<td>5.42</td>
<td>12 050</td>
<td>pDSBA3</td>
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<tr>
<td>FimD$_{NTD(1-125)}$</td>
<td>13655</td>
<td>125</td>
<td>4.41</td>
<td>6 090</td>
<td>pFimD-Nconst2AG</td>
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<tr>
<td>FimD$_{CTD(672-833)}$</td>
<td>17565</td>
<td>162</td>
<td>6.09</td>
<td>18 580</td>
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<td>FimC-FimI$_{ox}$</td>
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<td>8.92</td>
<td>38 520</td>
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<tr>
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<td>6.79</td>
<td>26 030</td>
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<tr>
<td>FimC-FimA$_{ct}$</td>
<td>54343</td>
<td>496</td>
<td>6.91</td>
<td>50 100</td>
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</tbody>
</table>

Table 8: A summary of all proteins used in this study and their properties. All protein properties: molecular weight (Mr), number of amino acids (#aa), isoelectric point (pI), extinction coefficients at 280 nm were calculated using Expasy-Prot Param. Table contains a list of all plasmids, which were used to express proteins needed in this thesis.
Materials and Methods

7.5. METHODS: Production and purification of proteins and protein complexes

7.5.1. Cytoplasmic expression of FimI, FimI<sub>t</sub> and FimA from inclusion bodies

Plasmids carrying genes fim<sub>I</sub>, fim<sub>I</sub><sub>t</sub> and fim<sub>A</sub> without a periplasmic signal sequence were used to overexpress proteins of type 1 pili, FimI, FimI<sub>t</sub> and FimA, in a cytoplasm in forms of inclusion bodies (IB). For production of FimI, electro-competent cells E.coli BL21 (DE3) (50 µl) (Studier and Moffatt 1986) were transformed with the plasmid pET11a-pfimI<sub>cyt</sub> (Ignatov 2009) (see Table 8). FimI<sub>t</sub> is a truncated variant of type 1 pilus subunit FimI, which lacks the first 20 N-terminal amino acid residues. For production of FimI<sub>t</sub> and FimA, E.coli BL21 (DE3) cells were transformed with the plasmid (pET11a-pfimI<sub>t</sub>) and (pET11a-pfimA<sub>wt-cyt</sub>), respectively. Bacterial cell cultures were grown in DYT medium supplemented with 0.1 mg/ml ampicillin (DYT/Amp) at 37 °C for 30 min and then plated onto LB plates containing 0.1 mg/ml ampicillin (LB/Amp). One single colony from each plates of transformants containing E.coli BL21 (DE3)/pET11a-pfimI<sub>cyt</sub>, E.coli BL21 (DE3)/pET11a-pfimI<sub>t</sub> and E.coli BL21 (DE3)/pET11a- pfimA<sub>wt-cyt</sub> cells were picked out after incubation at 37 °C in a thermostat overnight (o/n). The bacterial cultures of E.coli BL21 (DE3) harbouring appropriate plasmids were diluted 100 times (15 ml), transferred into 1.5 l DYT/Amp in 5 l flasks and shaking at 140 rpm at 37 °C till the cell cultures reached an OD<sub>600</sub> of 1. Gene expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were incubated for additional 4 hours at 140 rpm at 37 °C, sedimented by centrifugation at 5 000 rpm (Centrifuge GS3) for 20 min at 4 °C in 500 ml plastic flasks and pellets containing inclusion bodies were frozen at -20 °C.

IB obtained by cytosolic overexpression of FimI<sub>U_red</sub>, FimI<sub>t_red</sub> and FimA<sub>U_red</sub> in E.coli BL21 (DE3) were isolated as described (Rudolph and Lilie 1996). Pellets of bacterial cells were resuspended in resuspension buffer = 30 ml (100 mM NaH<sub>2</sub>PO<sub>4</sub>/NaOH pH 7.5, 150 mM NaCl) (3 ml/g of wet cells). Maximum cell lysis by high-pressure dispersion was achieved by passing the solution 3-5 times through a Microfluidizer M-110L (Microfluidics, USA) with a chamber pressure of 14 000 PSI. Disrupted cells were harvested by centrifugation at 20 000 rpm (SS34
rotor) for 20 min at 4 °C. To prevent cosedimentation of cell debris, IB isolates were resuspended in ≈ 25 ml of wash buffer containing detergent (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 2 % Triton-X-100). After 1-hour incubation at 4 °C in shaking conditions, IB were sedimented by centrifugation at 20 000 rpm (SS34 rotor) for 20 min (4 °C). To maximize cell lysis efficiency washing step of IB isolates was repeated two times. The rinsing step of IB was performed by removing detergent using 25 ml of 20 mM Tris/HCl, pH 8.0, followed by resuspension and incubation for 30 min mixing at 4 °C. IB were harvested by centrifugation at 20 000 rpm (SS34 rotor) for 20 min at 4 °C and the rinsing step was repeated again. Pellets of FimI<sub>U</sub><sup>red</sup>, FimI<sub>t</sub><sup>U</sup><sup>red</sup> and FimA<sub>U</sub><sup>red</sup> were almost white with some black traces and were stored at -20 °C. Because IB solubilization requires strong denaturants, pellets were resuspended and dissolved in 9 ml of Solubilization buffer (20 mM Tris/HCl, 6 M GdmCl (C-grade, Nigu), 1 mM EDTA, 20 mM DTT, pH 8.0). Solubilisation was performed for 2 hours mixing at 4 °C followed by centrifugation for 20 min at 50 000 rpm (MLA-80) at 4 °C. The supernatants containing FimI, FimI<sub>t</sub> and FimA were passed over a Superdex 200 26/60 gel filtration column equilibrated in 20 mM Acetic acid/NaOH pH 4.5, 6 M GdmCl was used to purify FimI<sub>U</sub><sup>red</sup>, FimI<sub>t</sub><sup>U</sup><sup>red</sup> and FimA<sub>U</sub><sup>red</sup>. To minimize formation of intramolecular disulfide bonds, proteins were diluted to a final concentration of 2-5 μM and CuCl₂ at final concentration of 1 μM was added to catalyze formation of the single disulfide bond by air oxidation. Air oxidation was performed at alkaline conditions (6 M GdmCl, 20 mM Tris/HCl pH 8.0) by mixing for 24-48 hours at 20 °C. Next, oxidized FimI<sub>U</sub><sup>ox</sup>, FimI<sub>t</sub><sup>U</sup><sup>ox</sup> and FimA<sub>U</sub><sup>ox</sup> were then concentrated using Vivaflo 50/200. To get rid of the wrongly oxidized forms of FimI<sub>U</sub><sup>ox</sup>, FimI<sub>t</sub><sup>U</sup><sup>ox</sup> and FimA<sub>U</sub><sup>ox</sup>, purification using Superdex 200 26/60 column equilibrated in 6 M GdmCl, 20 mM Tris/HCl pH 8.0 was used. For refolding, denatured purified FimI<sub>t</sub><sup>U</sup><sup>ox</sup> and FimI<sub>U</sub><sup>ox</sup> in 6 M GdmCl, 20 mM Tris/HCl pH 8.0 (≈ 300 μM) were rapidly diluted in 20 mM Acetic acid/NaOH pH 5.0 (RT) to a final concentration of 5 μM; the final concentration of GdmCl was ≤ 0.1M. Refolding was performed for 60 min at 4 °C followed by dialysis in 20 mM Acetic acid/NaOH pH 5.0 mixing at 4 °C o/n to get rid of remaining GdmCl. Refolded FimI<sub>ox</sub><sup>N</sup> and FimI<sub>t</sub><sup>ox</sup><sup>N</sup> were applied on a RESOURSE S 6 ml column (GE Healthcare,USA) equilibrated in 20 mM Acetic acid/NaOH pH 5.0. Refolded FimI<sub>ox</sub><sup>N</sup> and FimI<sub>t</sub><sup>ox</sup><sup>N</sup> were eluted at 300 mM NaCl using a linear gradient 0-
500 mM NaCl and identity of proteins were analysed by 15 % SDS-PAGE gel. The pulled fractions were dialyzed in 20 mM Tris/HCl pH 8.0 and applied to a Superdex 200 26/60 column equilibrated in 20 mM Tris/HCl pH 8.0, 150 mM NaCl.

Purified FimA\textsuperscript{Uox} was refolded by rapid dilution (1:30) in refolding buffer containing 50 mM Acetic acid/NaOH pH 4.5, 1 M NaCl followed by dialyzing against 2 x 5 l of 20 mM Tris/HCl pH 8.0 performed at 4 °C o/n. Then anion exchange chromatography using RESOURSE Q 6 ml was used to separate pure homogenic FimA\textsuperscript{Nox} from other FimA oligomeric species.

The concentration of pure FimI\textsuperscript{Nox}, FimI\textsuperscript{tNox} and FimA\textsuperscript{Nox} were determined by Spectrophorimeter (Agilent Technologies, USA) at 280 nm absorbance (\( \varepsilon_{280} \) FimI\textsuperscript{Nox} = 21 100 M\(^{-1}\) cm\(^{-1}\), \( \varepsilon_{280} \) FimI\textsuperscript{tNox} = 15 600 M\(^{-1}\) cm\(^{-1}\), \( \varepsilon_{280} \) FimA\textsuperscript{Nox} = 3110 M\(^{-1}\) cm\(^{-1}\)). Proteins were verified by electrospray ionization mass spectrometry (ESI-MS) (expected mass for FimI\textsuperscript{Nox} 17 169 Da; measured mass for FimI\textsuperscript{tNox} 17 169 Da, expected mass for FimI\textsuperscript{tNox} 15 012 Da; measured mass 15 012.5 Da, expected mass for FimA\textsuperscript{Nox} 15 827 Da; measured mass 15 825 Da). The aliquots were frozen in liquid nitrogen and stored at -80 °C.

7.5.2. Cytoplasmic expression and purification of FimC and FimC\textsubscript{yy}

For cytoplasmic overexpression of C-terminally (His) 6-tagged FimC, electrocompetent cells of \( E.\text{coli} \) BL21 (DE3) (Studier and Moffatt 1986) were transformed with a plasmid pFimC\textsubscript{cyt}. For cytoplasmic overexpression of spectroscopically silent FimC\textsubscript{cher} plasmid pfimC\textsubscript{cher} and competent cells \( E.\text{coli} \) BL21 (DE3) Tuner (see Table 7) were used. The first pre-culture was prepared by inoculating 4 ml of DYT containing 0.1 mg/ml Amp with a single colony of \( E.\text{coli} \) BL21 (DE3)/pFimC\textsubscript{cyt} and BL21 (DE3) Tuner/ pfimC\textsubscript{cher}. After 8-hours of incubation at 37 °C, 170 rpm, 150 µl of first pre-culture was used for inoculation of 150 ml DYT/Amp. The second pre-culture was incubated at 37 °C, 150 rpm o/n and used for the preparation of main culture 6 x 1.5 l of DYT/Amp in 5 l flasks. Dilution 1:100 of second pre-culture and
incubated was used to inoculate the main culture and incubated at 37 °C, 150 rpm until OD_{600} reached 1.0. The protein expression of FimC was induced by adding IPTG in a final concentration of 1 mM. After IPTG induction, the bacterial cells were incubated additional 4 hours and harvested for 10 min at 4200 g at 4 °C (Sorvall SLC-6000 rotor). For the preparation of soluble protein extract, pellets were resuspended in 50 mM NaH₂PO₄/NaOH pH 8.0, 300 mM NaCl, 1 mM EDTA) (3 ml/g) using ultraturrax (8000 rpm). The cells were disturbed by Microfluidizer and centrifuged for 45 min at 20 000 rpm (Sorvall SS34 rotor) at 4 °C. The soluble fraction containing FimC was dialyzed twice against 5 l of 50 mM NaH₂PO₄/NaOH pH 8.0, 300 mM NaCl at 4 °C. The first purification step was performed by 14 ml self-packed Ni²⁺-affinity column equilibrated in 50 mM NaH₂PO₄/NaOH pH 8.0, 300 mM NaCl. To elute histidine rich *E. coli* proteins a gradient of 8 % Buffer B was applied (Buffer B: 50 mM NaH₂PO₄-NaOH pH 8, 300 mM NaCl, 250 mM imidazole). To elute pure FimC a gradient of 8-100 % buffer B was used. Collected fractions of 8 ml per tube were analysed by 15 % SDS-PAGE gels and appropriate fractions were pulled for prominent peak corresponding to homogenic FimC. Then protein FimC was dialyzed 2 x against 5 l of 20 mM MOPS/NaOH pH 6.8 at 4 °C. Aggregated forms of FimC were removed by centrifugation at 20 000 rpm (SS34 rotor), 15 min at 4 °C. The next purification step was performed using cation exchange RESOURSE S 6 ml column (GE Healthcare, USA) at 4 °C. Running buffer containing 20 mM MOPS/NaOH pH 6.8 and B-buffer (Buffer A + 400 mM NaCl) were used to wash out potentially unbound proteins, 4 column volumes (CV) of buffer A was applied. Pure homogenic FimC was eluted by applying a gradient of 0-100 % buffer B over 25 CV, followed by 100 % buffer B for 5 CV. Aliquots of 3 ml collected fractions were analysed by 15 % SDS-PAGE gel. Fractions for pure FimC corresponding to the first peak were pulled and dialyzed against 5 l of 20 mM Tris/HCl pH 8.0 (RT) to remove present NaCl. The concentration of pure FimC was determined by Spectrophorimeter (Agilent Technologies, USA) at 280 nm absorbance (ε_{280} FimC= 22 860 M⁻¹ cm⁻¹, ε_{280} FimCyy= 14 250 M⁻¹ cm⁻¹). Purity of protein was verified by ESI MS (expected mass of FimC: 23 553 Da; measured mass 23 553.5 Da, expected mass of FimCyy: 23 507 Da; measured mass 23 507 Da). The aliquots were frozen in liquid nitrogen and stored at - 80 °C. Yield of pure FimC was 39 mg per liter of bacterial
cultures; total yield 350 mg and 18 mg per liter of bacterial culture; total yield 162 mg for FimC<sub>yyHis</sub>.

7.5.3. Preparation of complexes FimC-Fim<sub>Nox</sub>I and FimC-Fim<sub>Tox</sub>I

For refolding, stock solutions (=250-300 µM) of pure denatured oxidized Fim<sub>Uox</sub>I and Fim<sub>Tox</sub>I (see chapter 7.5.1) in 6 M GdmCl, 20 mM Tris/HCl pH 8.0 were refolded by rapid 1:60 dilution in 20 mM MOPS/NaOH, pH 6.7 and in the presence of a small molar excess of chaperone FimC (8 µM) (see chapter 7.5.2) over Fim<sub>Uox</sub>I / Fim<sub>Tox</sub>I (5 µM). Refolding was performed at 4 °C stirring for 1 hour and then concentrating using Vivaflow 50/200. To remove remaining GdmCl, protein-complexes were dialyzed against 5 l of 20 mM Mops/NaOH, pH 6.7 at 4 °C o/n. During dialyzing step, slightly aggregation was observed for FimC-Fim<sub>Nox</sub>I complex but huge aggregation for FimC-Fim<sub>Tox</sub>I complex. Aggregates were removed by centrifugation at 20 000 rpm (SS34 rotor) at 4° for 20 min and protein solution was passed over a RESOURSE S 6 ml column (GE Healthcare, USA) equilibrated in 20 mM MOPS/NaOH pH 7.0. To separate the FimC-Fim<sub>Nox</sub>I and FimC-Fim<sub>Tox</sub>I complex from the free chaperone FimC, a linear gradient from 0 to 0.3 M NaCl was applied. Protein complexes were eluted at around 150 mM NaCl. The fractions corresponding to FimC-Fim<sub>Nox</sub>I / FimC-Fim<sub>Tox</sub>I complexes were analysed by 15 % SDS-PAGE gel. Corresponding fractions were pulled and dialyzed 2 x against 5 l of 10 mM Tris/HCl pH 8.0. Final concentrations of protein complexes were determined by Spectrophorimeter (Agilent Technologies, USA) at absorbance 280 nm (ε<sub>280</sub> of FimC-Fim<sub>Nox</sub>I = 44 020 M<sup>-1</sup> cm<sup>-1</sup>, ε<sub>280</sub> of FimC-Fim<sub>Tox</sub>I = 38 520 M<sup>-1</sup> cm<sup>-1</sup>). Total yield of protein complexes were 5 mg and 22 mg for FimC-Fim<sub>Nox</sub>I and FimC-Fim<sub>Tox</sub>I, respectively. Complexes were frozen in liquid nitrogen and stored at -20 °C.

7.5.4. Periplasmic expression and purification of DsbA<sub>ox</sub>, DsbA<sub>yyox</sub>

Periplasmic DsbA containing a signal sequence was expressed and purified as previously described by Wunderlich & Glockshuber, 1993 (Wunderlich and Glockshuber 1993) and spectroscopically silent variant of DsbA<sub>yyox</sub> as described by (Hennecke et al. 1997). The
plasmid pDsbA3 was transformed into *E. coli* BL21 (DE3) pLysS cells (Pan and Malcolm 2000) and incubated for 30 min at 37 °C. The cell culture was streaked out on LB/Amp agar plate and after 8-hour incubation one colony was picked for inoculation of 60 ml DYT/Amp. The cell culture was incubated in 250 ml round-bottom flask at 27 °C shaking at 170 rpm o/n. The prepared preheated at 37 °C 3 x 1.5 l DYT/Amp mediums were each inoculated with 10 ml pre-culture and further were shaking at 160 rpm at 37 °C. When the cell cultures reached an OD\textsubscript{600} of 0.6, IPTG was added in a final concentration of 1 mM. The cells were harvested after 4 hours of incubation by centrifugation at 6 000 rpm g for 12 min. The pellets were frozen at -20 °C and used the next day. To thaw the pellets, they were kept on ice and then re-suspended in 45 ml of re-suspension buffer (200 mM Boric Acid/NaOH pH8.0, 160 mM NaCl and 5 mM EDTA supplemented with 100 mg/ml Polymyxine B Sulfate). The pellet suspension was stirred at 4 °C for 2 hours in a cold room, followed by centrifugation for 30 min at 30 000 rpm and the obtained supernatant was dialyzed against 5 l of 10 mM MOPS/NaOH pH 7.0 stirring at 4 °C o/n. Next day the protein solution was centrifuged for 12 min at 5000 x g and loaded onto RESOURCE Q 6 ml column (GE Healthcare, USA) pre-equilibrated with 10 mM MOPS/NaOH pH 7.0. Eluted peak fractions were analysed by 15 % SDS-PAGE gel. Fractions containing DsbA\textsubscript{ox} protein were applied to Superdex 200 26/60 column (GE Healthcare, USA) equilibrated in 20 mM MOPS/NaOH, 150 mM NaCl pH 7.0. After gel filtration, samples were analysed by 15 % SDS-PAGE, pulled and dialyzed o/n with stirring at 4 °C, 10 mM Acetic Acid/NaOH pH 4.0. The third purification step, was performed at RT using RESOURCE S 6 ml equilibrated with 10 mM Acetic Acid/NaOH pH 4.0 (Buffer A). DsbA\textsubscript{ox} was eluted by applying a gradient 0-100 % of 10 mM Acetic Acid/NaOH, 50 mM NaCl pH 4.0 (Buffer B). Eluted fractions were analysed by 15 % SDS-PAGE and fractions containing pure DsbA\textsubscript{ox} were pulled, dialyzed against 5 l of 20 mM MOPS/NaOH pH 7.0 stirring at 4 °C o/n. Protein concentration was determined at 280 nm (ε\textsubscript{280} of DsbA\textsubscript{ox} = 23 050 M\textsuperscript{-1} cm\textsuperscript{-1}), distributed into 1 ml aliquots, which were frozen in liquid nitrogen and stored at -20 °C until needed. The yield of pure DsbA\textsubscript{ox} was 16 mg per liter of bacterial culture; total yield 144 mg.
7.5.5. **Periplasmic expression and purification of FimD\textsubscript{NTD(1-125)}**

To express N-terminal domain of FimD (FimD\textsubscript{NTD(1-125)}) we used the original plasmid encoding N-terminal part of FimD residues 1-126 with its natural signal sequence to periplasm (Nishiyama et al. 2005). Since a glycine at position 126 is apparently a part of a flexible segment (residues 126–135) that binds the structured core to the transmembrane region of FimD, glycine was removed on this plasmid. The same construct (pfimDN construct 2 \(\Delta G\)) was used for preparation of FimC-FimH\textsubscript{p}-FimD\textsubscript{NTD(1-125)} (Nishiyama et al. 2005) and FimC-FimF\textsubscript{c}-FimD\textsubscript{NTD(1-125)} (Eidam et al. 2008). To overexpress FimD\textsubscript{NTD(1-125)} in periplasm, we used the protease-deficient *E. coli* HM125 (Meerman and Georgiou 1994). *E coli* HM125 were transformed with plasmid pfimDN construct 2 \(\Delta G\) and DYT/Amp plates were incubated at 30 °C o/n. Single colony was picked from a plate and inoculated into 4 ml DYT/Amp incubating at 30 °C at 200 rpm. The second pre-culture: 120 ml of DYT/Amp + 2.4 ml of first pre-culture (1:50 dilution). Dilution of 1:100 (15 ml) was used to inoculate 6 x 1.5 l of DYT/Amp with an overnight culture. Cells were grown at 30 °C 150 rpm till \(\text{OD}_{600}\) was 0.6, then IPTG at final concentration of 1 mM was added and cells were incubated for additional 4 hours. After incubation cells were harvested by centrifugation for 30 min at 10 000 rpm and 4 °C. Cell pellets were resuspended in the periplasmic extraction buffer containing 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mg/ml Polymyxine B Sulfate and 1 tablet of protease inhibitor cocktail. Extraction was performed for 2 hours at 150 rpm and 4 °C. Cell debris was removed by centrifugation for 30 min at 20 000 rpm (SS34 rotor) and 4 °C. Supernatant containing periplasmic FimD\textsubscript{NTD(1-125)} was dialyzed against 2 x 5 l of 20 mM Bis-Tris/HCl pH 6.2 at 4 °C. The first purification step was performed using QA52 column equilibrated in 20 mM Bis-Tris/HCl pH 6.2 at RT. The appropriate fractions were pulled and dialyzed against 2 x 5 l of 20 mM Bis-Tris/HCl pH 6.2. The second purification step was done with stronger anion exchanger column RESOURSE Q 6 ml at 4 °C. Fractions corresponding to the first prominent peak were pulled and dialyzed against 5 l of 20 mM Tris/HCl pH 8.0. For the last purification step, a column Superdex 75 26/60 equilibrated in 20 mM \(\text{NaH}_2\text{PO}_4/\text{NaOH}\) pH 7.4, 115 mM NaCl was used. Concentration of pure FimD\textsubscript{NTD(1-125)} was determined at 280 nm (\(\varepsilon_{280}=6090 \text{ M}^{-1} \text{ cm}^{-1}\)) and identity of FimD\textsubscript{NTD(1-125)} was verified by ESI-MS (calculated mass 13653 Da; measured mass 13653 Da). We purified FimD\textsubscript{NTD(1-125)} with
yields of 12.23 mg per liter of bacterial culture; total yield 111 mg. Aliquots of pure \( \text{FimD}_{\text{NTD}(1-125)} \) were frozen in liquid nitrogen and stored at -20 °C.

### 7.5.6. Purification of the ternary complex FimC – FimI \( t \) – FimD \( \text{NTD}(1-125) \)

FimC-FimI \( t \) complex and FimD \( \text{NTD}(1-125) \) were expressed and purified as described above (see chapters 7.5.3 and 7.5.5). The ternary complex was obtained by mixing 2.6 molar excess of pure FimD \( \text{NTD}(1-125) \) (60 µM) over FimC-FimI \( t \) (23 µM). The protein mixture was stirred for 1 hour at 4 °C and concentrated to approximately 10 ml by ultrafiltration at 4 °C using YM-10 membrane (Merck, Germany) with a nominal molecular weight limit of 10 kDa. Superdex 75 26/60 column (GE Healthcare, USA) equilibrated in 20 mM sodium phosphate pH 7.4, 115 mM NaCl was used to separate free FimD \( \text{NTD}(1-125) \) from FimC-FimI \( t \)–FimD \( \text{NTD}(1-125) \) complex. The first peak from an elution profile corresponded to a pure ternary complex FimC-FimI \( t \)–FimD \( \text{NTD}(1-125) \). The ternary complex was dialyzed against 5 l of 10 mM Tris/HCl pH 8.0 at 4 °C and concentrated to 19.5 mg/ml. \( \varepsilon_{280} \) of ternary complex FimC-FimI \( t \)–FimD \( \text{NTD}(1-125) \) = 44 600 M\(^{-1}\) cm\(^{-1}\).

### 7.6. Determination of protein concentration

Protein concentrations of pure proteins were calculated according to Gill & von Hippel (Gill and von Hippel 1989) based on measured absorbance at 280 nm and known molecular extinction coefficients at 280 nm: FimI \( U_{\text{ox}} \) \( \varepsilon_{280\text{nm}} \) = 21 100 M\(^{-1}\) cm\(^{-1}\), FimI \( U_{\text{ox}} \) \( \varepsilon_{280\text{nm}} \) = 15 600 M\(^{-1}\) cm\(^{-1}\), FimC \( \varepsilon_{280\text{nm}} \) = 22 860 M\(^{-1}\) cm\(^{-1}\), FimC \( \text{yyW36YW84YHis} \) \( \varepsilon_{280\text{nm}} \) = 14 250 M\(^{-1}\) cm\(^{-1}\), FimA \( U_{\text{ox}} \) \( \varepsilon_{280\text{nm}} \) = 3110 M\(^{-1}\) cm\(^{-1}\), DsbA \( \text{ox} \) \( \varepsilon_{280\text{nm}} \) = 23 050 M\(^{-1}\) cm\(^{-1}\), DsbA \( \text{yyW76FW126F} \) \( \varepsilon_{280\text{nm}} \) = 12 050 M\(^{-1}\) cm\(^{-1}\), FimD \( \text{NTD}(1-125) \) \( \varepsilon_{280\text{nm}} \) = 6090 M\(^{-1}\) cm\(^{-1}\), FimD \( \text{CTD}(673-833) \) \( \varepsilon_{280\text{nm}} \) = 18 580 M\(^{-1}\) cm\(^{-1}\). All absorption spectra were detected by Cary series UV-Visible Spectrophotometer (Agilent Technologies, USA).
7.7. Determination of sulfhydryl groups with DTNB

This is a standard protocol for the determination of free thiols (reaction of free SH groups in protein after mixing with DTNB) (Ellman 1959). To calculate the molarity of (-SH) groups in the assay, the absorbance needs to be measured and divide by the extinction coefficient of the reagent ($\varepsilon_{\text{TNB}} = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$). The molar extinction coefficient of TNB in water is constant in the pH range between pH 7.5- 8.5, where its thiol is in the deprotonated form.

Proteins of various concentrations (750 µl) were mixed with 4 x Test buffer (250 µl) (0.32 M NaH$_2$PO$_4$·H$_2$O pH 8.0, 2 mg/ml EDTA, 8.33 M GdmCl) at RT to get a total volume of 1000 µl. Analogous, reference sample devoid of protein and DTNB was used as a blank. Control 1 sample was prepared by mixing Test buffer and protein solution to determine $\varepsilon_{412\text{nm}}$ of protein for a standard curve. To determine $\varepsilon_{412\text{nm}}$ of DTNB, control sample 2 was prepared by mixing Test buffer, protein buffer and 30 µl of 10 mM DTNB. For measuring free SH reacted with DTNB and free DTNB at $\varepsilon_{412\text{nm}}$, a probe containing Test buffer and protein solution mixed with 30 µl 10 mM DTNB was prepared and incubated in dark at RT for 15 min. The sulfhydryl content of the target solution was determined by comparison with the obtained standard curve and probe. Extinction coefficients were detected by Cary series UV-Visible Spectrophotometer (Agilent Technologies, USA).

7.8. Far- and near-UV CD spectroscopy

Far-UV CD spectra were recorded using a J-715 spectropolarimeter (Jasco, USA) at 25 °C with a 0.1 cm cuvette and near-UV CD spectra with 1.0 cm quartz cuvette. Temperature, scanning speed, sensitivity, responds, bandwidth and data pitch were set to 25 °C, 20 nm/min, 100 mdeg/s, 0.5 s, 2 nm, 0.2 nm, respectively. The protein concentrations used in far-UV were 0.1 mg/ml and in near-UV 0.4 mg/ml. The monitored far-UV CD spectrum at 200-250 nm was subtracted by 20 mM Acetic acid/NaOH pH 5.0. The near-UV CD spectra for refolded FimI$^{\text{N,ox}}$ and unfolded FimI$^{\text{U,ox}}$ in 3 M GdmCl, 20 mM Acetic acid/NaOH pH 5.0 were measured at wavelength of 250-400 nm and subtracted by 20 mM Acetic acid/NaOH pH 5.0 and 3 M GdmCl, 20 mM Acetic acid/NaOH pH 5.0, respectively. All measured CD data containing the
row ellipticities were converted to mean residue ellipticities θ_{MRW} (deg. cm^2 dmol^{-1}) according to an equation described by Schmid (Schmid 1997).

7.9. Fluorescence spectroscopy

All fluorescence emission spectra were recorded in 20 mM Tris/HCl pH 8.0 at 25 °C recorded with a fluorescence spectrometer (temperature–controlled) Quantum Master 7 (PTI, USA) in 1.0 cm (emission path) x 0.2 cm (excitation path) quartz cuvettes. The spectra were measured at an excitation wavelength of 280 nm and emission wavelength between 280 and 450 nm. Bandwidth, scanning speed and response were set to 5 nm, 20 nm min^{-1}, 0.5 sec, respectively. Protein concentrations for recording spectra were 0.5 µM in 20 mM Tris/HCl pH 8.0 containing different concentrations of GdmCl. In an experiment, where stability of FimI_N in different salt concentration was measured, the fluorescence emission spectra were recorded in 20 mM Acetic acid/NaOH pH 5.0, 25 °C after 5 min incubation of FimI_N on ice. The mixture of FimI_U and FimC was produced by rapid 20x dilution of FimI_U (final GdmCl was 0.3 M) with refolding buffer containing FimC. Before recording each emission spectrum, protein solutions were centrifuged at 20 000 rpm, at 4 °C for 1 min. The setting of the parameters was kept the same for all measurements and each spectrum was subtracted by an adequate buffer condition.

7.10. Analytical fast ion-exchange chromatography (IEX)

Preparation of reduced FimI_U_red:

Denatured purified FimI_U (see chapter 7.5.1) in 3 M GdmCl, 20 mM Tris/HCl pH 8.0 was incubated with DTT at final concentration of 10 mM for 1 hour at 37 °C. To remove DTT, FimI_U_red was applied to a desalting Sephadex G-25 column equilibrated in 3 M GdmCl, 20 mM Tris/HCl pH 8.0, 0.5 mM EDTA. After desalting column, concentration of reduced FimI_U_red was determined by Spectrophotometer (see chapter 7.6) and reduced FimI_U_red was verified by Ellman’s assay (Ellman 1959) (see chapter 7.7).


**Importance of disulfide bond in FimC-FimI complex:**

Purified FimI\textsubscript{Uox} or FimI\textsubscript{Ured} (both at final concentrations of 2.5 µM) were incubated in the presence of a 2-fold molar excess of FimC (5 µM) for >5 min (RT) in 20 mM Tris/HCl pH 8.0. Final concentration of GdmCl in FimI\textsubscript{Uox}/FimI\textsubscript{Ured} after refolding with FimC was 0.27 M. A 12-fold molar excess of DsbA\textsubscript{Ox} (30 µM) over FimI\textsubscript{Ured} and 6-fold molar excess over FimC was added to the mixture of FimC/FimI\textsubscript{Ured} and incubated for >5 min (RT). Mixture of proteins (FimC/FimI\textsubscript{Ured}/DsbA\textsubscript{Ox}, FimC/FimI\textsubscript{Uox}, FimC/FimI\textsubscript{Ured}, FimC alone and FimI\textsubscript{Ured} and FimI\textsubscript{Uox}) were applied to a fast cation exchange chromatography at 4 °C using a 1 ml RESOURSE S column (GE Healthcare, USA) equilibrated in 20 mM MOPS/NaOH pH 7.0. Products of reactions were separated by a NaCl gradient 0-300 mM and peak intensities were detected at 230 nm.

**Kinetics of DsbA- and FimC-catalyzed folding of FimA and FimI:**

Reduced FimI\textsubscript{Ured} (2.5 µM) and FimA\textsubscript{Ured} (2.5 µM) were rapidly diluted 22 times in a mixture containing different concentrations of FimC/DsbA\textsubscript{Ox} in 20 mM Tris/HCl pH 8.0. Different concentrations are described by 3 conditions: condition A (5 µM DsbA\textsubscript{Ox} /2.5 µM FimC), condition B (2.5 µM DsbA\textsubscript{Ox}/5 µM FimC), condition C (5 µM DsbA\textsubscript{Ox}/5 µM FimC). The reaction mixture was incubated in 20 mM Tris/HCl pH 8.0 with a final concentration of 0.27 M GdmCl for 5 min (RT). After incubation the buffer was exchanged to 20 mM MOPS/NaCl pH 7.0 using Amicon MW 10 000. To separate reaction components a fast cation IEX at 4 °C using an analytical RESOURSE S 1 ml column (GE Healthcare, USA) equilibrated in 20 mM MOPS/NaCl pH 7.0 was applied. Formed products were separated with a NaCl linear gradient of 0-500 mM NaCl and detected at 280 nm. For better resolution, UV absorbance at 230 nm was used. The peaks in chromatograms were fitted and integrated using the Windows\textsuperscript{TM} software PeakFit\textsuperscript{TM} (SeaSolve Software Inc) and using the Exponentially Modified Gaussian (EMG) model. On the basis of individual peak areas obtained after IEX and the $\varepsilon_{230nm}$ of FimC and chaperone-complexes, the fractions of monomeric FimC, dimeric FimC-FimI-FimI, FimC-FimI, dimeric FimC-FimA-FimA and FimC-FimA were calculated. Only FimC bound in complexes FimC-FimI or FimC-FimA was calculated according to an equation describing calculated peak area of complex divided by $\varepsilon_{230nm}$ of complex and multiplied by $\varepsilon_{230nm}$ of...
FimC. The extinction coefficients at 230 nm ($\varepsilon_{230\text{nm}}$) for FimC and protein-complexes were as follows: FimC: 137 600 M$^{-1}$ cm$^{-1}$; FimC-FimI$^\text{N}$$^\text{ox}$ = 242 500 M$^{-1}$ cm$^{-1}$; FimC-FimA$^\text{N}$$^\text{ox}$ = 177 000 M$^{-1}$ cm$^{-1}$; FimC-FimI$^\text{N}$$^\text{ox}$-FimI$^\text{N}$$^\text{ox}$ = 347 500 M$^{-1}$ cm$^{-1}$; FimC-FimA$^\text{N}$$^\text{ox}$-FimA$^\text{N}$$^\text{ox}$ = 216 300 M$^{-1}$ cm$^{-1}$.

### 7.11. Analytical size exclusion chromatography (SEC)

To analyse the binding of FimC-FimI$_t$ to bind N-terminal domain of FimD$_{\text{NTD}(1-125)}$ we used analytical size exclusion chromatography (SEC). SEC is also often called molecular sieve, gel permeation or gel filtration chromatography. This analytical method separates molecules based on their molecular sizes and shapes by using the molecular sieve properties of a variety of porous materials. The Superdex 75 10/300 GL column (GE Healthcare, USA) column was used for separation of molecular masses of monomeric FimD$_{\text{NTD}(1-125)}$ ($M_w$=13.655 kDa), FimC-FimI$_t$ ($M_w$=38.567 kDa) and FimC-FimI$_t$-FimD$_{\text{NTD}(1-125)}$ complex ($M_w$=52.222 kDa). Based on standard molecular weight markers, which determines the fractionation range of the column, we were able to identify elution profiles of individual proteins or protein complexes. Concentrations of proteins used in this experiment were 20 µg, 60 µg and 80 µg for FimD$_{\text{NTD}(1-125)}$, FimC-FimI$_t$, FimC-FimI$_t$-FimD$_{\text{NTD}(1-125)}$, respectively. Proteins were eluted isocratically at RT in 20 mM Tris/HCl pH 8.0, 150 mM NaCl with a flow rate of 0.4 ml/min and volume of applied sample was 100 µl. Elution profiles of proteins were detected by measuring absorbance at 230 nm and evaluating using software SigmaPlot 12.2.

### 7.12. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of proteins after each purification step was determined by denaturing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) as described by (Laemmli 1970). The stock of 4 x SDS sample buffer was mixed with $\frac{1}{3}$ volume of sample containing proteins and incubated at 95 °C for $\approx$ 5 min prior to loading on a gel. For reducing gels, 4 x SDS reducing sample buffer (containing DTT) was used, while for
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oxidizing gels 4 x non-reducing sample buffer. Volumes of loaded samples were about 5-20 µl subjected to electrophoresis at limiting current of ≈ 35mA - 45mA for 1 hour. Coomassie Brilliant Blue G dye in 2 % solution was used to stain gels for 1 hour or o/n at RT.

7.13. Stopped-flow tryptophan fluorescence kinetics

All tryptophan fluorescence kinetics experiments were performed at pH 8.0, 25 °C on an SX18.MV stopped-flow instrument (Applied Photophysics). Tryptophan fluorescence change was recorded at the $\varepsilon_{ex}=280$ nm, using a glass filter with cut-off ≥ 320 nm in asymmetric (1:11) and symmetric (1:1) mixing conditions. In asymmetric mixing conditions syringe (2.5 ml) and syringe (250 µl) were used. Setting parameters for asymmetric mixing were: 2 bars, 4000 points, 20-100 seconds reaction time, temperature 25 °C, voltage 400 V, total drive volume 250 µl and slits ex: 3.5 nm and em: 10 nm. The final concentration of remaining GdmCl after mixing was 0.27 M. The same setting parameters, except of 6 bars pressure for symmetric mixing and 2.5 ml volume of both syringes, were used to measure stopped-flow fluorescence kinetics under the equimolar concentrations (1:1 mixing).

For DsbA-catalyzed oxidation kinetic experiments, FimI$_{U}^{ox}$ and FimI$_{t}^{U}^{ox}$ were reduced by 30 mM DTT incubating for 1 hour at 37 °C. After incubation, DTT was removed by desalting column Sephadex G-25 equilibrated in 3 M GdmCl, 20 mM Tris/HCl pH 8.0, 0.5 mM EDTA. To exclude air oxidation of FimI$_{red}$, FimI$_{t}^{U}^{red}$, FimA$_{red}$, all buffers were filtered (pore size 0.2 pm), de-gassed and immediately used for kinetic measurements.

Refolding experiments:

Oxidation of FimI$_{red}$, FimI$_{t}^{U}^{red}$ or FimA$_{red}$ by DsbA and FimC-catalyzed folding of FimI$_{ox}$, FimI$_{t}^{U}^{ox}$ or FimA$_{ox}$ were fitted according to second-order kinetics. Refolding of reduced subunit (X) in the presence of excess of oxidized DsbA and FimC was fitted according to a consecutive mechanism with two pseudo first-order rate constants (equations 1-3), where $\gamma_A$, $\gamma_B$, $\gamma_C$ represent the fractions of FimX$_{red}$, FimX$_{ox}$ and FimC-FimX, $k_1$ is the pseudo first-order rate constant of subunit oxidation and $k_2$ is the pseudo first-order rate constant of FimC-catalyzed folding.
\[ \gamma_A = \exp(-k_1 t) \]  
(1)

\[ \gamma_B = \frac{k_1}{k_2 - k_1} \{ \exp(-k_1 t) - \exp(-k_2 t) \} \]  
(2)

\[ \gamma_C = 1 + \frac{1}{k_1 - k_2} [k_2 \exp(-k_1 t) - k_1 \exp(-k_2 t)] \]  
(3)

Equation 4 and 5 were used to fit the observed fluorescence traces, where \( F_A \) corresponds to the fluorescence signal of the mixture of educts, \( F_C \) to the final fluorescence signal, and \( F_B \) corresponds to the signal of the mixture of oxidized, unfolded subunit, reduced DsbA and FimC.

\[ F_{obs} = F_A \gamma_A + F_B \gamma_B + F_C \gamma_C \]  
(4)

\[ F_{obs} = F_A \exp(-k_1 t) + F_B \left( \frac{k_1}{k_2 - k_1} \{ \exp(-k_1 t) - \exp(-k_2 t) \} \right) + F_C \left( 1 + \frac{1}{k_1 - k_2} [k_2 \exp(-k_1 t) - k_1 \exp(-k_2 t)] \right) \]  
(5)

### 7.14. Kinetic simulations

**Prediction of in vivo half-lives:**

Simulation of the kinetics of DsbA- and FimC-catalyzed oxidative folding of FimI and FimA *in vivo*. The fraction of \( \text{FimI}^{\text{U-red}} \), \( \text{FimI}^{\text{U-ox}} \) and the native FimC-FimI complex at periplasmic DsbA (86 \( \mu \)M) and FimC (23 \( \mu \)M) (Crespo et al. 2012) is plotted against folding time (t), assuming that DsbA and FimC are added at excess over \( \text{FimI}^{\text{U-red}} \) molecules. The simulations were analysed according to two consecutive irreversible reactions (see chapter 7.13) and *in vivo* half-life for both subunits, FimA and FimI, was determined.

**Confirmation of the catalysed folding rates of FimA and FimI with a kinetic competition assay:**

Three different reactions were simulated by Berkeley Madonna 8.3 program (Macey & Oster) in order to confirm measured kinetics results. Simulations are based on 4 following reactions described by numbers: (1) \( \text{FimA}^{\text{U-red}} (A) + \text{DsbA}_{\text{ox}} (B) \rightarrow \text{FimA}^{\text{U-ox}} (C) + \text{DsbA}_{\text{red}} (D) \), where \( k_1 \) represents an experimentally obtained second-order rate constant of DsbA-catalyzed oxidation of \( \text{FimA}^{\text{U-red}} \) (3.38 ± 0.01) \( \times 10^4 \) \( M^{-1} s^{-1} \) with initial concentrations
[A] = [FimA\textsubscript{red}] = 1 \mu M, [B] = [DsbA\textsubscript{ox}] = 1 \mu M. (2) simulating reaction: FimA\textsubscript{ox} (C) + FimC (D) \xrightarrow{k_2} \text{FimC-FimA}\textsubscript{ox} (E), where k_2 describes an experimentally obtained second-order-rate constant of FimC-catalyzed FimA\textsubscript{ox} (4.28 \pm 0.15) \times 10^4 \text{M}^{-1}\text{s}^{-1} with initial concentration [E] = [FimC] = 2 \mu M. (3) simulating reaction: FimI\textsubscript{red}(G) + DsbA\textsubscript{ox} (B) \xrightarrow{k_3} FimI\textsubscript{ox} (H) + DsbA\textsubscript{red} (D) using experimentally obtained second-order-rate constant of DsbA\textsubscript{ox}-catalyzed FimI\textsubscript{red} \ xrightarrow{k_3} (1.15 \times 10^6) \text{M}^{-1}\text{s}^{-1} with initial concentration of [G] = [FimI\textsubscript{red}] = 1 \mu M and [B] = [DsbA\textsubscript{ox}] = 1 \mu M. (4) simulating reaction: FimI\textsubscript{ox} (H) + FimC (E) \xrightarrow{k_4} \text{FimC-FimI}\textsubscript{ox} (I) using experimentally obtained second-order-rate constant of FimC-catalyzed FimI\textsubscript{ox} folding \ xrightarrow{k_4} (4.05 \pm 0.01) \times 10^4 \text{M}^{-1}\text{s}^{-1} with initial concentration [E] = [FimC] = 2 \mu M. Figure 17A, B and C illustrating simulated reactions were processed via SigmaPlot 12.2 software, where black line represents complex formation of FimC- FimA\textsubscript{ox} (E) and green line FimC-FimI\textsubscript{ox} (I).

7.15. Protein structure determination by X-ray crystallography

7.15.1. Crystallization

The purified protein complex FimC-FimI (22 mg/ml, 10 mM Tris/HCl pH 8.0) was used in crystallisation trials. The initial hit condition (4 M sodium formate, 0.1 M sodium cacodylate pH 6.5) was optimized in a 24-well crystallization setup by applying a gradient of pH (sodium cacodylate pH 5.5 – pH 7.0) and precipitant concentration (sodium formate 2.0 M to 4.5 M) to a mixture of 1.5 \mu l protein complex with 1 \mu l of mother liquor (ML) solution. The best crystallization condition was found to be 4.5 M sodium formate, 0.1 M sodium cacodylate at pH 6.5 (ML: 700 \mu l; drop: 2.5 \mu l). The complex was crystallized using the sitting drop vapour-diffusion method at 4 \degree C. First crystals were obtained after 3 days, cryo protected with 8 \mu l of 20% ethylene glycol in mother liquor solution directly added to the drop and cryo cooled in liquid nitrogen.

The most suitable crystallographic conditions for FimC-FimI-FimD\textsubscript{NTD\{1-125\}} complex were found by sparse matrix crystallisation screening at X-tal facility UZH Zürich. For optimization of conditions, we used 24-well plates with applied pH gradient of 0.1 M Hepes (pH 7.4 –
pH 8.4) combined with different precipitant types (PEG 4000, PEG 10000, PEG 5000MME, PEG 3350) in concentration ranges of 5% - 20%. Using the sitting drop vapour diffusion method, we obtained several crystals after 12 hours at 4 °C in presence of 1 µl ML containing 0.1 M Hepes pH 8.4, 15% PEG 4000 and 1 µl of FimC-Fiml_t-FimD_{NTD(1-125)} (19.5 mg/ml, 10 mM Tris/HCl pH 8.0) equilibrated against 700 µl reservoir solution. Crystals were cryo-protected in mother liquor containing 30% (v/v) ethylene glycol and flash-frozen in liquid nitrogen.

7.15.2. Data collection, processing and molecular replacement

Diffraction data for FimC-Fiml_t and FimC-Fiml_t-FimD_{NTD(1-125)} were collected at 100 K using a wavelength of 1 Å at beamline X06DA (Paul Scherrer Institute, Villigen, Switzerland) on a Pilatus 2MF pixel detector. For FimC-Fiml_t, data were processed to a final resolution of 1.75 Å with XDS (Kabsch 2010) in space group P 3 1 2 1 with one complex per asymmetric unit and cell dimensions of: a=166.22 Å, b=166.22 Å, c=51.44 Å, α=β=90°, γ=120°. From the total reflections (12 990), 1% were set aside as test reflections. For FimC-Fiml_t-FimD_{NTD(1-125)} data were also collected from a single crystal and processed to a final resolution of 1.70 Å. The obtained crystals have space group of P 2 1 2 1 and cell dimensions of crystals were: a=35.24 Å, b=104.16 Å, c=132.16 Å, α=β=γ=90°.

The structures were solved by molecular replacement using PHASER (McCoy et al. 2007). For FimC-Fiml_t, the binary complex of FimC-FimA_t (Protein Data Bank code 4DWH) was used as a search model. In the case of the ternary complex FimC-Fiml_t-FimD_{NTD(1-125)}, the FimC-Fiml_t complex was used as a search model.

7.15.3. Structure refinement

In FimC-Fiml_t structure refinement converged to the values of R_work = 0.164 and R_free = 0.187 (48.4–1.75 Å range) with good stereochemistry (see crystallographic table 5 for additional statistics). For the ternary structure, initial refinement after molecular replacement revealed the presence of FimD_{NTD(1-125)} which, as a result, was then copied from PDB 1ze3. The refinement converged to R_work and R_free of 0.204, 0.252 (48.5 – 1.7 Å range) respectively.
Both refinements were performed with PHENIX (Adams et al. 2010), COOT (Emsley et al. 2010) was used for manual rebuilding.
8. REFERENCES


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Ng TW, Akman L, Osisami M and Thanassi DG. The usher N terminus is the initial targeting site for chaperone-subunit complexes and participates in subsequent pilus biogenesis events. *Journal of Bacteriology*, 2004, 186: 5321-5331.


## 9. APPENDIX

### 9.1. List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>Angstrom or Ångström</td>
</tr>
<tr>
<td>a.u.</td>
<td>Arbitrary units (fluorescence spectroscopy)</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance units (UV-Vis spectroscopy)</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism Spectroscopy</td>
</tr>
<tr>
<td>CFD</td>
<td>FimC-FimF₁-FimD&lt;sub&gt;NTD(1-125)&lt;/sub&gt;</td>
</tr>
<tr>
<td>CHD</td>
<td>FimC-FimH&lt;sub&gt;p&lt;/sub&gt;-FimD&lt;sub&gt;NTD(1-125)&lt;/sub&gt;</td>
</tr>
<tr>
<td>CID</td>
<td>FimC-FimI&lt;sub&gt;t&lt;/sub&gt;-FimD&lt;sub&gt;NTD(1-125)&lt;/sub&gt;</td>
</tr>
<tr>
<td>CUP</td>
<td>Chaperone-usher pathway</td>
</tr>
<tr>
<td>CV</td>
<td>Column Volume</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>Dam</td>
<td>Deoxyadenosine methylase</td>
</tr>
<tr>
<td>DsbA&lt;sub&gt;ox&lt;/sub&gt;</td>
<td>Disulfide oxidoreductase, enzyme of Dsb (disulfide bond) family</td>
</tr>
<tr>
<td>DSC</td>
<td>Donor strand complementation</td>
</tr>
<tr>
<td>DSE</td>
<td>Donor strand exchange</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithio-bis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DYT/Amp</td>
<td>DYT medium containing ampicillin</td>
</tr>
<tr>
<td><em>E.coli</em></td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMG</td>
<td>Exponentially Modified Gaussian</td>
</tr>
<tr>
<td>ESI MS</td>
<td>Electrospray Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FimD&lt;sub&gt;CTD&lt;/sub&gt;</td>
<td>C-terminal domain of FimD (CTD)</td>
</tr>
<tr>
<td>FimD&lt;sub&gt;NTD&lt;/sub&gt;</td>
<td>N-terminal domain of FimD (NTD)</td>
</tr>
<tr>
<td>FimF₁</td>
<td>N-terminally truncated FimF</td>
</tr>
<tr>
<td>FimH&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Lectin-domain of FimH</td>
</tr>
<tr>
<td>FimH&lt;sub&gt;p&lt;/sub&gt;</td>
<td>Pilin-domain of FimH</td>
</tr>
<tr>
<td>FimI&lt;sub&gt;t&lt;/sub&gt;</td>
<td>N-terminally truncated FimI</td>
</tr>
<tr>
<td>GdmCl</td>
<td>Guanidiniumchlorid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IB</td>
<td>Inclusion bodies</td>
</tr>
<tr>
<td>IEX</td>
<td>Ion Exchange Chromatography</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRL</td>
<td>Left inverted repeats</td>
</tr>
<tr>
<td>IRR</td>
<td>Right inverted repeats</td>
</tr>
<tr>
<td>IRs</td>
<td>Inverted repeats</td>
</tr>
<tr>
<td>k</td>
<td>rate constant</td>
</tr>
<tr>
<td>k&lt;sub&gt;app&lt;/sub&gt;</td>
<td>apparent rate constant</td>
</tr>
<tr>
<td>Lrp</td>
<td>Leucine-responsive regulatory protein</td>
</tr>
<tr>
<td>ML</td>
<td>mother liquor</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-morpholino-propanesulfonic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>o/n</td>
<td>over night</td>
</tr>
<tr>
<td>ox</td>
<td>oxidized</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>pap</td>
<td>pyelonephritis-associated pilus</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PLPN</td>
<td>Plasmid library place number</td>
</tr>
<tr>
<td>PSI</td>
<td>Paul Scherrer Institute (Villigen, CH)</td>
</tr>
<tr>
<td>PSI</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>r.m.s.d.</td>
<td>root mean squared deviation</td>
</tr>
<tr>
<td>red</td>
<td>reduced</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-amino-2-(hydroxymethyl) propane-1,3-diol</td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic E.coli</td>
</tr>
<tr>
<td>UTIs</td>
<td>Urinary tract infections</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>XDS</td>
<td>X-ray diffuse scattering</td>
</tr>
</tbody>
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Marital status: married

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