The importance of the periplasmic protein FimC for the assistance of type 1 pilus assembly in Escherichia coli

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The importance of the periplasmic protein FimC for the assistance of type 1 pilus assembly in Escherichia coli

A dissertation submitted to the
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for the degree of
Doctor of Natural Science

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# Table of Contents

1 SUMMARY

2 ZUSAMMENFASSUNG

3 INTRODUCTION

3.1 BACTERIAL SURFACE ORGANELLES

3.2 P PILI AND TYPE 1 PILI OF *Escherichia coli*

3.2.1 Morphology

3.2.2 Genetic Organization

3.2.3 Regulation of Pilus Biosynthesis

3.3 Pilus Biosynthesis

3.3.1 Protein Secretion into the Periplasm

3.3.2 Chaperone-Usher Pathway

3.3.3 Three-Dimensional Structure of Pilus Chaperones

3.3.4 Families of Molecular Chaperones

3.3.5 Chaperone-Dependent Pilus Assembly

3.3.6 Pilus Formation at the Outer Membrane

3.4 Biological Role of Type 1 Pili

3.5 Aim of the Thesis

4 RESULTS

4.1 Characterization of FimC, a Periplasmic Assembly Factor for Biogenesis of Type 1 Pili in *Escherichia coli*

4.1.1 Abstract

4.1.2 Introduction

4.1.3 Experimental Procedures

4.1.3.1 Materials

4.1.3.2 Construction of expression plasmids
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.3.3</td>
<td>Expression and purification of FimC and its C-terminal domain</td>
<td>27</td>
</tr>
<tr>
<td>4.1.3.4</td>
<td>Protein concentration</td>
<td>28</td>
</tr>
<tr>
<td>4.1.3.5</td>
<td>Analytical gel filtration</td>
<td>29</td>
</tr>
<tr>
<td>4.1.3.6</td>
<td>Circular dichroism and fluorescence spectroscopy</td>
<td>29</td>
</tr>
<tr>
<td>4.1.3.7</td>
<td>Urea-induced unfolding equilibria and stopped-flow measurements</td>
<td>29</td>
</tr>
<tr>
<td>4.1.3.8</td>
<td>Peptide binding measurements</td>
<td>30</td>
</tr>
<tr>
<td>4.1.4</td>
<td>ACKNOWLEDGEMENTS</td>
<td>31</td>
</tr>
<tr>
<td>4.1.5</td>
<td>RESULTS</td>
<td>32</td>
</tr>
<tr>
<td>4.1.5.1</td>
<td>Expression and purification of FimC and its C-terminal domain</td>
<td>32</td>
</tr>
<tr>
<td>4.1.5.2</td>
<td>Folding and thermodynamic stability of FimC and its isolated C-terminal domain</td>
<td>32</td>
</tr>
<tr>
<td>4.1.5.3</td>
<td>FimC only weakly binds to the C-terminal peptide of the type 1 pilus adhesin FimH</td>
<td>33</td>
</tr>
<tr>
<td>4.1.6</td>
<td>DISCUSSION</td>
<td>35</td>
</tr>
<tr>
<td>4.1.7</td>
<td>REFERENCES</td>
<td>44</td>
</tr>
<tr>
<td>4.2</td>
<td>CHAPERONE-CONTROLLED STOICHIOMETRY OF A MACROMOLECULAR ASSEMBLY*</td>
<td>47</td>
</tr>
<tr>
<td>4.2.1</td>
<td>SUMMARY</td>
<td>48</td>
</tr>
<tr>
<td>4.2.2</td>
<td>INTRODUCTION</td>
<td>49</td>
</tr>
<tr>
<td>4.2.3</td>
<td>EXPERIMENTAL PROCEDURES</td>
<td>52</td>
</tr>
<tr>
<td>4.2.3.1</td>
<td>Reagents</td>
<td>52</td>
</tr>
<tr>
<td>4.2.3.2</td>
<td>General methods</td>
<td>52</td>
</tr>
<tr>
<td>4.2.3.3</td>
<td>Random mutagenesis on different FimC residues and construction of expression plasmids</td>
<td>52</td>
</tr>
<tr>
<td>4.2.3.4</td>
<td>Construction of the strain W3110ΔfimC</td>
<td>53</td>
</tr>
<tr>
<td>4.2.3.5</td>
<td>Screening for active and inactive FimC variants by agglutination with yeast cells</td>
<td>54</td>
</tr>
<tr>
<td>4.2.3.6</td>
<td>Detection of periplasmic FimC concentrations by immunoblotting</td>
<td>54</td>
</tr>
<tr>
<td>4.2.3.7</td>
<td>Electron microscopy</td>
<td>54</td>
</tr>
<tr>
<td>4.2.4</td>
<td>ACKNOWLEDGEMENTS</td>
<td>56</td>
</tr>
<tr>
<td>4.2.5</td>
<td>RESULTS AND DISCUSSION</td>
<td>57</td>
</tr>
<tr>
<td>4.2.5.1</td>
<td>Random mutagenesis of nine distinct FimC residues</td>
<td>57</td>
</tr>
</tbody>
</table>
4.2.5.2 Amino acid replacements in the FimC subunit binding site affect type 1 pilus assembly but do not necessarily abolish the overall chaperone function 59
4.2.5.3 There is no overall correspondence between agglutination intensity and FimC expression 60
4.2.5.4 Glutamate 80 replacements mainly reduce the FimC stability 60
4.2.5.5 The residue R116 is essential for the formation of the interdomain salt bridge 60
4.2.5.6 Pilus formation requires FimC concentration above a certain level 61
4.2.5.7 Agglutination efficiency does not correlate with pilus phenotypes 62
4.2.5.8 Mannose recognition does not require FimH integration into the pilus 62
4.2.5.9 Differences in pilus length correlate with homogeneity of assembled pili 63
4.2.5.10 FimC contact area is formed by residues differently involved in subunit recognition 64

4.2.6 REFERENCES 77

5 DISCUSSION 82

6 REFERENCES 88

7 APPENDIX 100

8 CURRICULUM VITAE 104

9 DANKSAGUNG 105
1 SUMMARY

The molecular interactions that occur during the attachment of pathogenic bacteria to host tissues are critical factors for the establishment of bacterial infections. These interactions are often mediated by specific organelles on the bacterial surface called pili. Pili are filamentous, hetero-polymeric protein structures that present receptor-specific molecules, which are required for recognition of and binding to host cell surfaces. About 70–80% of all so far isolated *Escherichia coli* strains may produce type 1 pili that enable them to cause uropathogenic diseases, like cystitis and urethritis. One bacterial cell may form up to 500 of these appendages, which are 0.5–2 μm long and 7 nm wide. Type 1 pili consist of 500–3000 pilus subunits, where 98% of all subunits comprise the main structural subunit FimA. FimA subunits assemble to a right-handed helix pilus rod that contains the adhesive protein FimH on the tip and in regular distances along the pilus. The adhesin specifically recognizes α-D-mannose moieties in receptors on host cell surfaces and hence mediates bacterial adhesion for the establishment of the first critical step in bacterial infection.

The biogenesis of type 1 pili is dependent on the 22.7 kDa periplasmic chaperone protein FimC. It is supposed to contribute to subunit folding and forms stable chaperone-subunit complexes in the periplasm, thereby preventing spontaneous, premature subunit polymerization. Moreover, FimC is assumed to deliver the pilus proteins to the assembly platform FimD in the outer membrane, which allows the translocation of pilus subunits to the cell surface and anchors the pilus to the bacterial membrane.

This thesis deals with the biochemical characterization of the type 1 pilus chaperone FimC and the investigation of the chaperone function during pilus assembly.

FimC is a 205–residue protein consisting of two immunoglobulin–like domains. In the first part of the thesis, FimC and its C-terminal domain were purified with high yields from an overproducing *E. coli* strain. The N-terminal domain proved to be extremely unstable and could not be overproduced.

Urea-induced equilibrium unfolding transitions at pH 7.0 and 25 °C, followed by fluorescence, revealed a free energy of folding of −38 kJ/mol for FimC, and a dramatically decreased stability of −4 kJ/mol for the isolated C-terminal domain. Consequently, the C-terminal domain in FimC is strongly stabilized by intramolecular interactions with the N-terminal domain. Inter-domain contacts thus provide the strongest contributions to the overall stability of FimC. This could be confirmed by strongly destabilizing effects of amino acid
replacements removing the invariant inter-domain salt bridge R116-D192. Of the three variants R116D, D192R and R116D/D192R, only the FimC variant D192R could be expressed in the periplasm, albeit with significantly lower yields compared to the wild type.

In contrast to the N-terminal FimC domain, the C-terminal domain lacks cis-prolines so that analysis of its folding kinetics allowed the verification of the two-state model of folding with stopped-flow fluorescence experiments. The equilibrium data could be confirmed by the kinetic measurements, which revealed rate constants of folding and unfolding of 15 s^{-1} and 3.4 s^{-1}, respectively.

The second part of this thesis focuses on the interaction between FimC and the structural pilus subunits. It had been suggested that bacterial pilus chaperones specifically recognize the individual pilus subunits via their conserved C-terminal octapeptide segments. However, surface plasmon resonance experiments showed that the affinity of FimC toward a peptide corresponding to the 11 C-terminal residues of the adhesin FimH is only very weak (K_D = 8 \mu M). In contrast, the complex between FimC and the intact adhesin FimH proved to be extremely stable, with at least 1000-fold lower dissociation constant. This demonstrates that bacterial pilus chaperones, unlike other chaperones, specifically interact with folded target proteins.

To test the in vivo function of FimC, a fimC null mutant of the E. coli K21 wild type strain W3110 was constructed. Surprisingly, the fimC deficient strain exhibits even 2-fold longer pili than the wild type, but the number of pili per cell is dramatically reduced from 100-500 to less than 10. This suggests that FimC is primarily important for the initiation of pilus assembly and possibly less critical for pilus growth.

In order to characterize the in vivo function of FimC in more detail, nine distinct residues in the subunit binding site of FimC were randomized, and 1400 of the resulting FimC variants were tested for their ability of complementing FimC deficiency in the fimC null strain. More than 600 FimC variants were sequenced, and 116 variants were characterized in detail with respect to recovery of protein expression and pilus phenotype of resulting bacteria. Most of the FimC variants dramatically affected the number of pili per cell, as well as the length and subunit composition of the pili. This extensive analysis revealed that bacterial pilus chaperones have fine-tuned affinities for the different pilus subunits, and that these affinities regulate both the initiation of pilus assembly and pilus stoichiometry.
ZUSAMMENFASSUNG


Die Biogenese von Typ 1 Pili ist abhängig von dem 22.7 kDa großen periplasmatischen Chaperon-Protein FimC. Es wird vermutet, daß es einen Beitrag zur Faltung der Pilusuntereinheiten leistet, und es bildet stabile Komplexe mit den Untereinheiten im Periplasma, was eine spontane, frühzeitige Polymerisierung der Untereinheiten verhindert. Außerdem wird angenommen, daß FimC die Pilusproteine zu der Assemblierungsplattform in der äußeren Membran, FimD, transportiert, welche die Pilusuntereinheiten zur Zelloberfläche transportiert und den Pili in der Bakterienmembran verankert.

Diese Arbeit beschäftigt sich mit der biochemischen Charakterisierung des Typ 1 Pilus-Chaperons FimC und Untersuchungen zur Funktion von FimC während der Pilusbildung.

Zusammenfassung


Im Vergleich zur N-terminalen Domäne enthält die C-terminale keine cis-Proline, sodaß die Analyse ihrer Faltungskinetik die Verifizierung des Zwei-Zustand-Modells der Faltung durch stopped-flow Fluoreszenzmessungen erlaubt. Die Gleichgewichtsdaten können durch kinetische Daten bestätigt werden, welche Geschwindigkeitskonstanten von $15 \, s^{-1}$ und $3.4 \, s^{-1}$ für die Faltung und Enfaltung ergaben.


Um die in vivo Funktion von FimC zu untersuchen, wurde eine FimC Nullmutante des E. coli K12 Wildtypstammes W3110 hergestellt. Überraschenderweise bildet der fimC knock-out Stamm sogar doppelt so lange Pili wie der Wildtyp, wobei aber die Zahl der Pili pro Zelle von 100–500 Pili auf weniger als 10 reduziert ist. Dies läßt vermuten, daß FimC in erster Linie für die Initiation der Pilusassemblierung wichtig ist, jedoch weniger kritisch für die Pilusverlängerung.

Um die in vivo Funktion von FimC noch detaillierter zu charakterisieren, wurden 9 spezielle FimC–Reste der Bindungsfläche für die Untereinheiten variiert und 1400 der resultierenden FimC Varianten auf ihre Eigenschaft untersucht, die FimC Abwesenheit zu kompensieren. Über 600 FimC–Varianten wurden sequenziert und 116 im Detail auf Proteinexpression und Phänotyp der resultierenden Bakterien überprüft. Die meisten der FimC–Varianten beeinflußten sowohl die Anzahl der Pili pro Zelle als auch die Länge und
die Zusammensetzung der Pili. Diese erweiterten Untersuchungen demonstrieren, daß bakterielle Pilus-Chaperone fein abgestimmte Affinitäten für unterschiedliche Pilusuntereinheiten besitzen und daß diese Affinitäten sowohl die Pilusinitiation als auch die Pilusstöchiometrie regulieren.
3 INTRODUCTION

3.1 Bacterial surface organelles

Organelle development is a fundamental process in all living cells. Pathogenic bacteria assemble virulence structures on their surfaces that direct their attachment to host tissues (Klemm and Krogfelt, 1994). Specialized molecules on bacterial surfaces, called adhesins, allow the recognition of receptor molecules on host cells with stereochemical specificity. This molecular interaction between bacteria and their hosts plays a crucial role in the colonization of host tissue by bacteria and the subsequent establishment of an infection. In many instances, adhesins are presented in hair-like structures called pili or fimbriae that extend from the bacterial surface. Obviously, pili serve as pedestals for adhesin presentation and thus favor the microbial attack on epithelial cells. Pili or fimbriae are long filamentous polymeric protein structures of different morphologies. Usually, fibers having a rod-like morphology with a diameter of approximately 7 nm are called pili. All Gram-negative bacterial species that have been examined so far as well as few Gram-positive microbes like corynebacteria, possess one or more types of adhesive pili or fimbriae; (Krogfelt, 1991; Mol and Oudega, 1996). Many clinical E. coli isolates also produce curli that are thin, irregular and highly aggregated surface structures (Olsen, 1989). In other cases, adhesins are directly associated with the bacterial membrane and are thus termed non-pilus adhesins which have been found in E. coli or Heliobacter pylori (Hultgren et al., 1993; Schmidt, 1994).

In addition to differences in morphology, adhesin-presenting appendages may be classified with respect to their adhesive properties. Besides their ‘natural’ receptors, many adhesins also bind to erythrocytes of various animal species. The adhesin-mediated agglutination of red blood cells may or may not be blocked by D-mannose and is therefore called mannose-sensitive or mannose-resistant hemagglutination, respectively (Mol and Oudega, 1996).

Another classification of pili is based on their assembly pathway. Bacterial pilus assembly comprises the fine-tuned of subunits coordination for hetero-oligomeric pilus formation, the correct incorporation of individual subunits into the pilus and prevention of premature association between intrinsically assembly-competent subunits. The process of organelle assembly may be divided into four classes: I) the chaperone-usher pathway (type I; P; S; F1C; K88 pili) II) the extracellular nucleation-precipitation pathway (curli) (III) the general secretion pathway (type IV; bundle forming pili) and IV) the alternate chaperone pathway/atypical assembly pathway (CS1 pili) (Hultgren et al., 1996; Soto and Hultgren, 1999).
As previously stated, there is a strong correlation between bacterial adhesion and pathogenesis. For example *E. coli* is the most common bacterial species causing urinary tract infections (UTI) in human. Such infections may manifest themselves as acute cystitis, urethritis or more severely as pyelonephritis that are caused by type 1 or P pili, respectively. Other typical adhesin-mediated diseases are diarrhea and meningitis (Levine et al., 1983; Hultgren et al., 1985; Hultgren et al., 1996).

This thesis focuses on pili that are assembled via the chaperone-usher pathway, especially on type 1 pili of *Escherichia coli*.

### 3.2 P pili and type 1 pili of *Escherichia coli*

#### 3.2.1 Morphology

P pili are composite fibers that form a right-handed helix, approximately 7 nm in diameter and 1 μm in length, containing 3.28 subunits per turn. The pilus rod, formed by the main subunit PapA, has a 1.5 nm helical cavity winding through the rod. According to a three-dimensional reconstruction of P pili, at least four interactions of PapA in the pilus rod are observed. Two of them are between neighboring molecules as a head-to-tail contact that define the fibrillar polymer. Two additional interactions are formed between one molecule and another three subunits behind as well as one three subunits ahead which coil the polymer into a right-handed helix (Bullitt and Makowski, 1998; Bullitt and Makowski, 1995; Gong and Makowski, 1992). On the distal end of each pilus rod a flexible adhesive fibrillum or pilus tip was found that predominantly comprised repeating subunits of PapE, arranged in an open helical conformation (Kuehn et al., 1992; Lindberg et al., 1987). The adhesin PapG mediates the binding to Galα(1,4)Gal moieties on glycolipids of uroepithelial cells. It is joined to the fibrillum via a specialized adapter protein PapF, whereas the adhesin-containing tip is linked to the pilus rod by another adapter protein, PapK (Jacob-Dubuisson et al., 1993a). Finally, incorporation of the minor subunit PapH was found to terminate the pilus growth by functioning, together with the PapC usher, as the pilus anchor, connecting P pili to the bacterial cell (Baga et al., 1987). Interestingly, overexpression of the adapter protein PapK leads to production of pili with shorter tips, whereas overexpression of PapH results in shortened pilus rods (Jacob-Dubuisson et al., 1993b).

Like P pili, type 1 pili of are heteropolymeric protein structures that are expressed by 70-80% of all isolated *Escherichia coli* strains. One single bacteria cell may contain 100-500 of
these filamentous appendages that are 1-2 μm long (Brinton, 1965). Type 1 pili also exhibit a rod-shaped structure, predominantly formed by the 15.8 kDa structural protein FimA. It is arranged in a right-handed helix with 3.125 residues per turn, with an axial hole of 2.0-2.5 nm and an external diameter of 6-7 nm (McMichael and Ou, 1979; Chick et al., 1981; Orndorff and Falkow; 1985). The adhesive moiety of type 1 pili was reported to be the 29.1 kDa subunit FimH that specifically binds to α-D-mannose-containing receptors of certain host tissues (Minion et al., 1986; Hanson et al., 1988; Klemm et al., 1990; Krogfelt, 1991).

![Figure 1. Electron micrograph of an E. coli W3110 cell with type 1 pili (kindly provided by E. Hahn and P. Wild).](image)

The localization of FimH within the pilus is controversially discussed. It may either be located separated from the rod in a fibrillar tip (Hanson and Brinton, 1988; Jones et al., 1995), exclusively exist in regular distances along the pilus shaft (Krogfelt et al., 1990) or ubiquitous within the pilus (Abraham et al., 1988; Ponniah et al., 1991; Klemm and Krogfelt, 1994;). Structural integration of FimH is probably facilitated by two other minor components FimF and FimG that additionally have been found to be crucial for regulation of number and length of type 1 pili (Klemm and Christiansen, 1987; Russell and Orndorff, 1992). The fibrillar tip structure of type 1 pili is presumably formed by FimG and FimH and probably also by FimF, and located similarly to P pili, on the distal end of the pilus rod (Jones et al., 1995). The individual components of type 1 pili are linked by hydrophobic and hydrophilic interactions by forming a very rigid and stable structure.
Type 1 pili are resistant to proteolysis by trypsin as well as stable against various denaturing agents, like SDS or 6.0 M urea (Salit and Gotschlich, 1977; Jones et al., 1993). Their dissociation into monomeric subunits is solely possible after incubation at 37 °C in saturated guanidine chloride for several hours (Eshdat et al., 1981) or by boiling the pili at pH values below 1.8 (Brinton, 1965; McMichael and Ou, 1979). This extraordinary stability of type 1 pili may be required for maintenance of the pilus structure in the specific environment of host tissues, such as urine during bladder infection.

Eshdat et al. (1981) have demonstrated that in vitro re-assembly of dissociated structural subunits into type 1 pili requires bivalent ions like Mg²⁺. Moreover, pili composed of FimA, FimF, FimG and FimH tend to break and depolymerize in contrast to pili consisting solely of FimA subunits suggesting that introduction of the minor subunits disturbs and destabilizes the regular array of FimA (Krogfelt and Klemm, 1988; Klemm and Krogfelt, 1994).

3.2.2 Genetic organization

P pili represent the best-characterized bacterial pilus system. Genes involved in biogenesis and expression of functional P pili, called pap (pyelonephritis-associated pili) are clustered in an operon structure on the chromosome of uropathogenic E. coli (Hull et al., 1981). The pap gene cluster is found in DNA segments in which genes for piliation are linked to genes for multiple virulence factors (Swenson et al., 1996; Hung and Hultgren, 1998). Six of the 11 pap genes encode structural proteins: PapA, the main subunit of P pili, PapH, a proposed pilus anchor; PapK, the adapter that links the tip fibrillum to the pilus rod; PapE, the predominant protein of the tip; PapF, the adapter joining the adhesin to the tip fibrillum and PapG, the P pilus adhesin (Hull et al., 1981). Downstream of the structural genes and immediately in vicinity to the gene of the main structural subunit, there is found a pair of genes, papI and papB that is responsible for regulation of pilus expression (Hultgren et al., 1996). In the middle of the operon two genes are located whose gene products are not part of the pilus. The papD gene codes for a periplasmic protein being essential for pilus assembly and therefore is called pilus chaperone. The papC gene product is located in the outer membrane and forms the assembly platform anchoring the pili to the bacterial surface. The role of the papJ gene is not clear, it is suggested to act as a co-chaperone (Tennent et al., 1990).

The genetic organization of the type 1 pilus gene cluster is quite similar to the pap operon, although only nine genes are required for pilus biogenesis (Figure 2). Two of them, fimB and fimE, code for cytoplasmic located recombinases involved in the regulation of type 1 pilus expression (Klemm, 1986; Mcclain et al., 1991). The adjacent gene encodes the main
structural pilus subunit FimA (Klemm, 1984; Orndorff and Falkow, 1984). Downstream of fimA, the fiml gene is found encoding a protein that shows high homology to FimA, although its function is not clear. Adjacentely, two genes are located, fimC and fimD, coding for proteins that are not part of the pilus but have a crucial role for pilus assembly. Hence, FimC, the periplasmic pilus chaperone facilitates subunit incorporation into the pilus (Klemm, 1992; Jones et al., 1993;), whereas FimD, a pore forming protein enables the translocation of newly formed type 1 pili through the outer membrane and anchors them in the bacterial membrane (Klemm and Christiansen, 1990). On the distal part of the gene cluster three genes are located, fimF, fimG and fimH encoding the minor subunits of type 1 pili including the mannose specific adhesin FimH (Klemm and Christiansen, 1987). As illustrated in Figure 2, there are five promoters in the gene cluster, two individual for fimB and fimE, one responsible for the expression of the major subunit encoding gene, fimA, fiml and fimC chaperone. In the fimC gene a promoter is found that controls the fimD expression. Expression of the genes for the minor subunits fimF, fimG and fimH is achieved by a single promoter existing within fimD ensuring a 1:1:1 mRNA level (Olsen and Klemm, 1994).

Figure 2. Arrangement of the fim genes in the genome of E. coli K12 wild type. Sizes (in kDa) of the individual gene products including the signal sequence (black segments) are indicated. Arrows represent promoter regions (Klemm et al., 1994).

3.2.3 Regulation of pilus biosynthesis

Since regulation of pilus expression has been studied extensively for type 1 pili, this chapter mainly focuses on this pilus system (Figure 3). The expression of type 1 pili in E. coli was found to be under phase variation control since bacterial cells show fluctuations in their
phenotype and are either piliated or bald. This phase variation is accomplished by a 314 bp invertible DNA segment upstream of the major structural subunit FimA that contains the promoter responsible for expression of fimA fimI and fimC. By inversion of this so-called switch, the orientation of the fimA promotor is reversed and consequently individual cells may switch between piliated and non-piliated phases (Abraham et al., 1985; Eisenstein, 1981). Switch inversion requires the fim recombinases FimB and FimE (Klemm, 1986; Mcclain et al., 1991; Gally et al., 1996) and additional accessory proteins. In the absence of both the fimB and fimE gene, the invertible element becomes locked in either “on” or “off” orientation (Pallesen et al., 1989). The two recombinases were found to have mainly an antagonistic role, in the way that FimB confers mostly an “on” and FimE an “off” orientation of the switch (Blomfield et al., 1991; Gally et al., 1996).

Knock-out studies and DNA homology analysis revealed that integration host factor (IHF) is required for DNA switch inversion since it binds to the DNA segment flanking the fim switch (Dorman and Higgins, 1987; Eisenstein et al., 1987; Blomfield et al., 1997). Furthermore, the nucleoid-associated protein (H-NS) influences the rate of fimA promoter inversion, and mutations in the hns gene result in a 100-fold increased inversion rate (Olsen and Klemm, 1994).

An additional factor was identified, the leucine-responsive regulatory protein (Lrp), that is a site-specific DNA-binding protein and involved in expression of type 1 pili as well as P pili (Braaten et al., 1992; Blomfield et al., 1993; Roesch and Blomfield, 1998; van der Woude et al., 1996). Lrp stimulates the fim inversion by binding to two core sites within the switch which also overlaps with an IHF binding site, suggesting a possible interaction between Lrp and IHF (Gally et al., 1993; Gally et al., 1996).

Moreover, leuX was identified as another regulatory gene of type 1 pilus expression that encodes a minor leucine tRNA specific for the codon UUG. Since the fim switch requires the two recombinases FimB and FimE that contain six and two UUG codons, respectively, a limited amount of LeuX could favor the translation of FimB relative to FimE. Thereby the invertible DNA element is kept in the “off” position (Burghoff et al., 1993; Newman et al., 1994; Ritter et al., 1997).

Besides the above mentioned factors, the formation of pili is strictly dependent on bacterial growth conditions like temperature and medium. The fimE-promoted switching from “on” to “off” occurs more rapidly at lower temperature, whereas at typical mammalian body temperature, (37 - 41°C) the highest probability of switching from “off” to “on” occurs (Gally et al., 1993; Olsen et al., 1998).
In the case of P pili, regulation of pilus expression via an invertible DNA segment is similar to that of type 1 pili, but there are more regulatory factors. For example, binding of Lrp to the pap DNA appears to inhibit methylation of these sites by Dam, that specifically methylates GATC sequences. Since there are five methylation sites, pap transcription is quite sensitive for Dam activities, that may either stimulate or repress pap transcription (Blyn et al., 1990; Braaten et al., 1994).

Furthermore, specific post-transcriptional processing of pap mRNA was reported (Naureckiene and Uhlin, 1996; Nilsson et al., 1996). The papB gene coding for a regulatory protein and papA encoding the main pilus subunit are co-transcribed and subsequent endonucleolytic cleavage results in rapid decay of papB mRNA and long-lived papA mRNA.

Consequently, regulation of pilus expression involves a multitude of factors that influence the normal spontaneous DNA switching. These mechanisms enable bacteria to respond to environmental signals and to change their phenotypes in either a piliated, adhesive or a bald, non-adhesive state. This transition normally occurs with a frequency of $10^{-3}$ per generation (Eisenstein, 1981).
3.3 Pilus biosynthesis

3.3.1 Protein secretion into the periplasm

For the formation of pilus structures on bacterial surfaces production and transport of a sizable number of proteins from the cytoplasm to the outer membrane must be guaranteed. With exception of the regulatory proteins, all pilus subunits, the chaperone and the assembly platform are synthesized as precursors with an amino-terminal signal. After their translocation through the periplasmic membrane via the sec-apparatus the 25-30 amino acids signal peptides are cleaved off and proteins are released into the periplasmic space (Dodd and Eisenstein, 1984; Pugsley, 1993; Jacob-Dubuisson et al., 1994b). All subunits of type 1 and P pili contain disulfide bridges, while the type 1 pilus chaperone FimC lacks cysteine residues. Efficient folding of pilus subunits as well as folding of PapD, that is the only member of pilus chaperones containing a disulfide bond, requires the periplasmic thiol/disulphide oxidoreductase DsbA, the main catalyst of disulfide bond formation in the periplasm (Jacob-Dubuisson et al., 1994a). The finding that strains deficient in dsbA still assemble type 1 pili, albeit less efficiently, but cannot produce P pili, implicates that oxidation of pilus subunits can either proceed spontaneously or is mediated by another oxidoreductase like DsbC (Hultgren et al., 1996).

3.3.2 Chaperone-usher pathway

Pilus assembly is a highly controlled process, ensuring that every pilus has virtually the same subunit stoichiometry and overall structure. The process of subunit assembly and pilus secretion across the outer membrane is independent on cellular energy and must therefore be thermodynamically driven (Jacob-Dubuisson et al., 1994b). Thus, correct incorporation of various pilus components seems to be dictated by factors participating in their assembly. Two specialized families of proteins that are not part of the pilus structure were described to be essential for pilus formation, periplasmic chaperones and the outer membrane anchor proteins, called ushers (Norgren et al., 1987; Lindberg et al., 1989; Klemm and Christiansen, 1990; Klemm, 1992). These factors represent the junction in the pathway of the subunit assembly, connecting subunit release into the periplasm and a well-ordered pilus structure at the outer membrane. Polypeptide chains of pilus subunits entering the periplasm have to fold correctly and have to target loci in the outer membrane where pilus formation occurs at the right time and in a polymerization-competent manner. Pilus chaperones are proposed to assist in these processes by forming specific complexes with individual pilus proteins.
Except for the adhesins, different pilus subunits show similarities of up to 60% in their amino acid sequences. In the N-terminal as well as C-terminal region they exhibit segments with high homology. These findings together with their comparable sizes give rise to the assumption that pilus subunits have identical three-dimensional structures and possibly similar association patterns. Thus, homologous regions may play an important role for subunit-subunit contact within the pilus or for interactions with the chaperone and the usher. However, different incorporation rates might be realized by different expression levels of the subunits and a special graduated affinity to the chaperone and the usher (Kuehn et al., 1993; Soto et al., 1998; Soto and Hultgren, 1999). For the P pilus adhesin PapG, both highly conserved amino acid stretches were found, even though the sequence corresponding to the N-terminal pilin region was in the middle of the protein. This assumes a pilin-like structure extended by an N-terminal part responsible for receptor recognition (Hultgren et al., 1989; Haslam et al., 1994).

The chaperone-usher pathway represents a model for a directed fine-tuned assembly of about thousand proteins that end up in a polymeric, highly ordered functional organelle outside of the bacterial cell. The importance of a chaperone for this process was proved by corresponding gene deletion resulting in a non-piliated phenotype in P and type 1 pili. It could be shown that pilus subunits collapse in absence of the chaperone into off-pathway aggregates which targets them for proteolytic degradation (Lindberg et al., 1989; Jones et al., 1993; Bullitt et al., 1996). For the P pilus system it was found that aggregation of pilus subunits in the periplasm leads to activation of two parallel pathways: the Cpx two-component signaling system and the σE modulatory pathway, that increase the transcription of the gene encoding the periplasmic protease DegP (Danese and Silhavy, 1997; Pogliano et al., 1997). There are several lines of evidence that pilus chaperones may form bimolecular complexes with subunits emerged from the periplasmic membrane (Lindberg et al., 1989; Hultgren et al., 1993; Hultgren et al., 1996). This is also supported by the finding that pilus subunits expressed in the periplasm were stabilized by co-expression with their chaperones (Tewari et al., 1993). Interestingly, PapA, the main subunit of P pili, was detected as a 2:1 complex with PapD that questions the strict formation of heterodimeric chaperone-subunit complexes during subunit assembly into a pilus structure (Striker et al., 1994). After successfully catching pilus proteins in a chaperone complex, the subunit has to pass through the periplasm to reach the assembly platform FimD in the outer membrane. Here, the complex needs to dissociate under release and concurrent incorporation of the subunit into the pilus by interacting with other subunits and possibly with the assembly platform. Consequently, the
unbound chaperone is then again available for new incoming subunits. In summary, the chaperone-usher model suggests several functions for pilus chaperones: i) They specifically recognize unfolded subunits in the periplasm, assist in their correct folding at the chaperone surface and thus prevent subunit degradation. ii) After subunit folding chaperones cap matured interactive surfaces and thereby prevent non-productive subunit polymerization in the periplasm and keep them in an assembly-competent state. iii) Chaperones transport bound pilus components to the outer membrane located ushers and guide their release to the growing pilus under dissociation of the chaperone-subunit complex. iv) Due to different affinities to the individual pilus proteins, chaperones determine the stoichiometry of the mature pilus.

3.3.3 Three-dimensional structure of pilus chaperones

Structural information about factors that mainly control the process of pilus formation, like chaperones, help to understand molecular mechanisms of protein-protein interactions independent of any energy source. The three-dimensional structures of the pilus chaperones PapD and FimC were solved by X-ray crystallography (Holmgren and Brändén, 1989) and NMR, respectively (Pellecchia et al., 1998). A database search yielded 31 proteins of bacteria with sequence similarity to these chaperones, and may thereby be included in the family of bacterial periplasmic chaperones, even though they facilitate the assembly of pili of entirely different morphologies (Hung et al., 1996). The pilus chaperones are classified into two subgroups on the basis of their sequences, namely the rod-like fiber forming chaperones and chaperones that assemble non-pilus adhesins or very thin fibers. However, it could be shown that in all of these proteins several residues are still invariant or conservatively substituted and thus showing 25-56% identity to PapD. Additionally, they all possess a molecular weight of about 25 kDa (Holmgren et al., 1992; Hung et al., 1996; Bonci et al., 1997). Hence, for all periplasmic chaperones, a similar globular fold and a comparable mechanism of assistance in subunit assembly are suggested. They all have a conserved hydrophobic core that maintains the overall immuno globulin-like feature of their two domains (Williams and Barclay, 1988).

PapD and FimC share 34% sequence identity and have a very similar three-dimensional structure, consisting of two globular domains that are arranged in a boomerang-shaped structure. In FimC, the two domains show antiparallel β-sheets arranged in a β-barrel and a β-sandwich for the N-terminal and C-terminal domain, respectively. Both domains are connected by a 15-residue linker forming a short 310-helix (Pellecchia et al., 1998). The cleft between the domains contains surface-exposed residues, highly conserved or invariant throughout the entire superfamily. Only in part these residues are shown to be involved in
recognition of structural subunits (Slonin et al., 1992). Many of them make intramolecular domain-domain contacts and thereby stabilize the chaperone structure (Holmgren et al., 1992). In case of the latter the charged salt bridge formed by E80, R116 and D192 was shown to be relevant for the orientation of the domains that, is somewhat different to that in PapD. The main differences between the two proteins were found in the C-terminal domain that possesses an additional β-strand in PapD, which is connected to the preceding strand by a disulfide bond. Due to the high structural homology of the N-terminal domains of PapD and FimC, this region is supposed to bind pilus subunits in a comparable manner. In contrast, the C-terminal domains are assumed to interact with the membrane-bound usher during subunit delivery. The correspondence of conserved residues to their location in the three-dimensional structures of the FimC and PapD suggest a general functional mechanism for all members of this family of pilus chaperones. This is supported by the finding that a recombinant molecule consisting of the PapD N-terminal domain and the FimC C-terminal domain forms complexes with PapD subunits but is not able to interact with the P pilus usher PapC (Jones et al., 1997). (Klemm et al., 1994) could show that the FimC-FimD assembly machinery recognizes minor subunits of F1C pili that also were incorporated into type 1 pili by forming hybrid organelles. The exchange of one of the two assembly factors, FimC or FimD, by the corresponding protein of F1C pili was not successful. This indicates a high pairwise specificity for ushers and their correct chaperones that guaranties incorporation of subunits into the right pilus system (Klemm et al., 1995).
3.3.4 Families of molecular chaperones

In contrast to pilus chaperones that exclusively assist in pilus formation, proteins known as molecular chaperones act differently, mainly by inhibition of aggregation during protein folding. Although protein folding happens spontaneously and is determined by the amino acid sequence and the environmental conditions, molecular chaperones were found often to be required for the correct folding of polypeptide chains. Under special conditions like osmotic or heat shock, molecular chaperones are expressed in increased levels. They are often found to be associated to aggregated or misfolded proteins carrying several diseases. They have been defined as a group of unrelated proteins mediating the correct folding of other proteins but are not themselves components of the final structure. These chaperones inhibit protein aggregation and increase protein re-folding efficiency in an ATP-dependent mechanism (Hartl, 1996; Beissinger and Buchner, 1998).

The best-characterized chaperones are the proteins belonging to the Hsp70 family and the chaperonin or GroE family. Hsp70 binds to short, linear segments of an unfolded protein containing hydrophobic residues. Binding of ATP to the chaperone and hydrolysis in conjunction with other co-chaperones defines its affinity state for the substrates. In the GroE system, cooperation of the two components, GroEL and GroES, allows the folding of non-native proteins of 10-55 kDa by encapsulation in a chaperone cage (Hartl, 1996; Beissinger and Buchner, 1998). Thus, in contrast to periplasmic pilus chaperones, which are suggested to interact in an energy-independent manner with different pilus proteins, molecular chaperones bind unspecifically to unfolded, partially folded or even aggregated proteins and mediate their correct folding by ATP hydrolysis.

3.3.5 Chaperone-dependent pilus assembly

The molecular mechanisms of subunit recognition and binding by pilus chaperones, the transfer of subunits to the outer membrane as well as the final step of subunit assembly into a pilus is not completely understood. The isolation of the PapD-PapG and the FimC-FimH complexes from the periplasm demonstrated that these subunits are stable associated with the chaperones (Hultgren et al., 1989; Kuehn et al., 1991; Pellecchia et al., 1999). Two observations provide the basis for studies of the interaction between pilus subunits and their chaperones: i) there are highly conserved residues in all members of the chaperone superfamily and ii) there are high similarities between the C-terminal segments of all pilus subunits from rod-like pilus structures. This suggests an overall common binding mechanism.
FimF VTAGHINATATFTLEYQ
FimI VTCGIANAQAWFSLTYQ
FimA ATPGAANADATFKVQYQ
FimG ATQGTIQAVISITYTYS
FimH VTAGNVQSIIGVTFVYQ

**Figure 4.** Alignment of the 17 C-terminal residues of type 1 pilus structural subunits. Amino acids that are invariant or conserved among all subunits of rod-like forming pili are bold indicated.

As shown in Figure 4, type 1 pilus subunits possess a motif with invariant tyrosine, glycine and threonine residues corresponding to position 2, 14 and 16 from the C-terminus, respectively. Furthermore, an intervening sequence of alternating hydrophobic amino acids can be observed.

For P pili it was shown that deletion of the 14 carboxyl terminal residues of the adhesin PapG abolished PapD binding and PapD-PapG complex formation in vivo (Hultgren et al., 1989). In addition it was demonstrated by ELISA binding assays that even point mutations in the C-terminal region of PapG at positions 2, 4, 6 and 8 from the COOH-terminus negatively affect the interaction to PapD (Soto et al., 1998). Based on this information, a set of synthetic peptides corresponding to the C-terminal segments of P pilus subunits was tested for their ability to bind to PapD. It was demonstrated that PapD binds best to the COOH-terminal 19 amino acids of PapG and, albeit with reduced affinity, to comparable peptides of PapE, PapF and PapK (Kuehn et al., 1993). Moreover, the length of the peptides was shown to control the binding to PapD, since at least the last 7 carboxyl terminal residues were required for a detectable interaction. To gain insight into the molecular basis of the PapD-PapG interaction, PapD was co-crystallized with the C-terminal 19-residue peptide of PapG and PapK (Kuehn et al., 1993). The peptides bound in an extended conformation along a β-strand of the N-terminal PapD domain with the carboxyl terminus anchored in the domain cleft via charged hydrogen bonds to the invariant residues R8 and K112. Together with the finding that modification of the free C-terminus by amidation leads to loss of binding, the crucial role of the charged COOH-terminus for peptide binding is obvious. Site-directed mutations in R8 and K112 abolished PapD binding to PapG in vitro and lead to a hemagglutination-negative phenotype, whereas the overall structure of PapD is not affected. This directly demonstrates the importance of the invariant cleft residues R8 and K112 for substrate binding (Slonim et al., 1992; Kuehn et al., 1993). According to these data, a general binding model was
suggested in which subunits interact with their C-terminal segments with the chaperone by extending one of the β-sheets of the N-terminal domain by so-called β zippering. Hence, specificity of subunit recognition was proposed to be given mainly by the alternating pattern of hydrophobic C-terminal residues. Since (Kuehn et al., 1991) could show that PapD is able to bind to reduced, denatured PapG which restores the PapD-PapG complex, recognition of non-native subunits and subsequent folding on the chaperone surface was assumed. In this process, PapD may provide a platform for β-strand zippering, allowing the subunits to achieve their native-like conformation. (Soto et al., 1998) even discussed triggering of the subunit import into the periplasm by the chaperone via β-zippering and their parallel release from the periplasmic membrane.

Whereas the important role of conserved subunit COOH-terminal amino acids for intermolecular subunit-chaperone contact could be shown, it is likely that even more residues are involved in that process. So, non rod-like pilus subunits show no sequence homology at the C-terminus but their assembly is also mediated by related chaperones (Hultgren et al., 1996). This gives support to a probable role of the C-terminal segments of subunits for subunit-subunit interactions that define the rod-like structure of the mature pilus.

### 3.3.6 Pilus formation at the outer membrane

Besides binding of subunits to the chaperone, the second critical factor for pilus biosynthesis is the high-molecular-weight assembly platform in the outer membrane, also named usher. It is mainly involved in conversion of chaperone-subunit complexes into bacterial appendages. The number of pili formed per cell is directly related to the amount of usher produced (Hultgren et al., 1991). FimD knock-out studies revealed a non-piliated phenotype and accumulation of the chaperone-subunit complex in the periplasm. In contrast, overexpression of FimD in a strain lacking the fim gene cluster resulted in cell lysis, which could be repealed by coexpression of pilus subunits (Klemm and Christiansen, 1990; Jacob-Dubuisson et al., 1994b). This demonstrates the importance of the usher for pilus expression. Furthermore, there is a hint for the formation of outer membrane pores achieving subunit translocation across the outer membrane. By high-resolution electron microscopy, the P pilus usher, PapC was found to assemble into ring-shaped complexes of at least 6 subunits containing central pores of 2 nm in diameter (Thanassi et al., 1998b). Ushers are predicted to have a high β-strand content, typical for bacterial outer membrane proteins, and are likely to present large periplasmic regions for the interaction with the chaperone-subunit complexes (Thanassi et al., 1998a). In comparison to translocation of proteins across the inner membrane as completely
unfolded polypeptide chains, the usher has to interact specifically with folded pilus subunits, since they are bound to the chaperone in a highly folded conformation.

Electron microscopy experiments proved the growth of pili from the base (Lowe et al., 1987), which implies that subunits have to be presented on the chaperone surface in a way that allows an effective interaction with the last subunit incorporated into the pilus. Furthermore, there is an indication that subunits found on the distal tip of the pilus are favored for the interaction with the usher and thus initiate pilus assembly. *In vitro* studies proved the chaperone and chaperone-subunit complexes of type 1 and P pili to interact with the usher (Dodson et al., 1993; Saulino et al., 1998). It could be shown that ushers form ternary complexes with chaperone-subunit complexes with different affinities. The chaperone in complex with the adhesin was found to bind with the highest affinity to the usher and real-time kinetics performed by surface plasmon resonance revealed this complex to bind in the tightest and fastest way (Saulino et al., 1998). Therefore it is suggested that pilus ushers regulate the ordered targeting of chaperone-subunit complexes to the outer membrane assembly site, where the chaperone is dissociated from the respective subunit, allowing their polymerization into the pilus. Moreover, dissociation rates for all chaperone-subunit complexes were found to be slow, which might be due to subunit arrangement in the growing pilus. Since the usher-forming pore has a diameter of 2-3 nm pilus subunits can not be translocated through the outer membrane in a helical arrangement like in the pilus rod that is 6.8 nm wide. Therefore subunits may be translocated across the outer membrane as an extended protein chain out of 2 nm similar to the tip fibrillum. At the very outside of the bacterial surface it certainly adopts its helical conformation which may drive the subunit passage through the membrane (Thanassi et al., 1998b). This is in good agreement with the finding that pilus rods may be unraveled into linear fibers by glycerol (Abraham et al., 1992). Consequently, the usher has an active role in pilus assembly by allowing subunit transport to the bacterial surface and defining the subunit localization in the mature pilus.

### 3.4 Biological role of type 1 pili

Pili are organelles of attachment that mediate bacterial colonization of host cells and therefore are pathogenicity factors. It recently could be shown that type 1 pili specifically recognize uroplakin Ia and Ib, two major membrane glycoproteins of the urothelia apical plaques (Wu et al., 1996; Mulvey et al., 1998). Anchorage of *E. coli* to host surfaces via type 1 pilus-uroplakin interaction may play a critical role in bladder colonization. Binding of the tip adhesin to epithelial cells may principally influence the outward growth of the pilus, since
shortened pili were found after adhesion (Mulvey et al., 1998). Even pilus retraction after
attachment was proposed, which would cause a more intimate contact of the bacteria to the
uroplakin coated cells and subsequent exfoliation of the host bladder cells as a defense
mechanism. For bacteria, this would result in accumulation of unassembled subunits in the
periplasm that, besides other factors, may activate the Cpx pathway. That up-regulates the
expression of the periplasmic protease DegP as well as a number of other factors like the
disulfide oxidoreductase DsbA or cis-trans prolyl isomerases (Danese and Silhavy, 1997;
Jones et al., 1997; Pogliano et al., 1997).

In addition, Baorto et al. (1997) reported that type 1 pili are able to mediate survival and
long-term persistence of bacterial strains inside macrophages. Therefore, bacteria bearing
type 1 pili represent frequent pathogens that can cause spontaneous recurrent infections. In
this context, Langermann et al. (1997) have shown that FimH, the mannose-binding protein
subunit of type 1 pili is a promising vaccine to prevent mucosal infections in *E. coli*. 
3.5 Aim of the thesis

This thesis focuses on the type 1 pilus chaperone FimC, especially on its \textit{in vitro} behavior and its assistance in assembly of pilus subunits.

FimC should be biochemical characterized as a model protein for pilus chaperones since it shows high homology to a number of other pilus chaperones. In this context, protein folding and thermodynamic stability against denaturants should be serve as a prerequisite for future studies especially for the interaction with pilus subunits. Since the three-dimensional structure of FimC, solved by NMR revealed two globular domains, isolated domains should be analyzed for their function in stabilizing the protein structure and assisting in pilus formation. Furthermore, it was interesting to investigate the highly conserved buried interdomain salt bridge by \textit{in vivo} and \textit{in vitro} experiments to prove its role for protein stability and chaperone function.

In the second part of this thesis elimination of the gene encoding the chaperone and analysis of the resulting strain should broaden the understanding of chaperone mediated pilus assembly. In addition, a genetic screening system for active \textit{fimC} mutants on the basis of plasmid-coding complementation of \textit{fimC} deficiency should be established. In respect to this, it should be interesting to randomize distinct FimC residues and to test the resulting variants for their contribution in chaperone binding to pilus proteins during the pilus biosynthesis.
4 RESULTS

4.1 Characterization of FimC, a periplasmic assembly factor for biogenesis of type 1 pili in *Escherichia coli*

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4.1.1 Abstract

Assembly of type 1 pili from *Escherichia coli* is mediated by FimC, a periplasmic chaperone with two immunoglobulin-like domains. FimC is assumed to recognize the individual pilus subunits in the periplasm mainly via their conserved C-terminal segments, and to deliver the subunits to an assembly platform in the outer membrane. Here we present the first biochemical characterization of a periplasmic pilus chaperone and analyze the importance of the two chaperone domains for stability and function. Comparison of the isolated C-terminal domain with wild type FimC revealed a strongly reduced thermodynamic stability, indicating strong inter-domain interactions. The affinity of FimC toward a peptide corresponding to the 11 C-terminal residues of the type 1 pilus adhesin FimH is at least 1000-fold lower compared to binding of intact FimH, confirming that bacterial pilus chaperones, unlike other chaperones, specifically interact with folded pilus subunits.

*Keywords*: type 1 pili/FimC/periplasmic pilus chaperones/protein folding and assembly/protein stability
4.1.2 Introduction

An important initial event during the establishment of infections by pathogenic Gram-negative enterobacteria is the attachment of bacteria to host cell receptors. This process is mediated by adhesive surface organelles termed pili or fimbriae (1; 2; 3; 4). Pili are large, hetero-oligomeric protein filaments anchored to the bacterial outer membrane. Type 1 pili play an important role for the uropathogenicity of Escherichia coli strains. They enable the bacteria to bind to mannose-containing receptors at host cell surfaces (3) and enhance the virulence of pathogenic E. coli strains (5; 6). Binding of type 1 pili to host cell receptors is mediated by the adhesin FimH, a pilus subunit found at the tip and possibly in several copies along the pilus (7; 8; 9; 10; 11). In addition, type 1 pili also mediate survival and long-term persistence of uropathogenic E. coli strains inside macrophages and may thus contribute to the spontaneous reoccurrence of E. coli infections such as cystitis (5). For these reasons, type 1 pili are potential targets for the development of antibiotics, which, for example, prevent pilus assembly or inhibit adhesiveness. FimH also constitutes a promising vaccine for prevention of mucosal E. coli infections (12; 13). Type 1 pili are 0.5–2 μm long and 7 nm wide filaments (3) composed of 500–3000 protein subunits. Overall, 9 different E. coli proteins are involved in the biogenesis of type 1 pili, whose genes are clustered at 98 minutes of the E. coli K-12 chromosome (3; 14). FimA, the main structural pilus subunit, comprises about 98% of all pilus proteins, while the residual 2 percent are comprised by FimF, FimG, the adhesin FimH and possibly FimI (3). The outer membrane protein FimD anchors the pilus to the bacterial surface and represents its assembly platform, while the cytosolic proteins FimB and FimE regulate the transcription of pilus genes (15). Type 1 pilus assembly in vivo depends on FimC, a monomeric assembly factor of 205 amino acids in the periplasm (16; 17). FimC is assumed to form stoichiometric complexes with each of the five different subunits in the periplasm and to deliver the subunits to FimD. This oligomeric outer membrane protein allows translocation of the pilus subunits to the cell surface and their incorporation into the growing pilus (1; 3; 4; 18; 19; 20). The three-dimensional structure of FimC in solution has been solved by nuclear magnetic resonance (NMR) spectroscopy (21) (Figure 1). The structure is similar to the crystal structure of the P pilus chaperone PapD (22) that shares 34% sequence identity with FimC. FimC consists of two globular domains (residues 1–115 and 131–205) with an immunoglobulin-like tertiary structure that are connected by a short linker peptide (residues 116–130) (21). Previous X-ray studies on complexes between PapD and synthetic peptides corresponding to the C-terminal residues of P pilus subunits PapG and
PapK indicated that the conserved C-terminal octapeptide sequences of bacterial pilus subunits are an important common recognition motif of bacterial pilus chaperones (23; 24). In these PapD/peptide complexes, the peptides are bound in an extended conformation to the N-terminal PapD domain in a β-sheet-like manner via hydrogen bonds. Their C-terminal carboxylates form charged hydrogen bonds with the invariant chaperone residues Arg8 and Lys112 (23; 24). However, NMR studies on the 1:1 complex between FimC and the intact adhesin FimH have shown that the contact area between FimC and FimH is much larger than that identified in the structures of the PapD/peptide complexes (25). Specifically, there are approximately 30 additional residues from the N-terminal FimC domain that interact with FimH and do not correspond to peptide-binding residues in PapD. The recently solved X-ray structure of the FimC-FimH complex from *E. coli* has shown that FimH is a two-domain protein. The N-terminal domain contains the mannose binding site of the adhesin, whereas the C-terminal domain exhibits homology to the other structural pilus subunits and possesses an immunoglobulin-like fold. FimC only binds to the C-terminal FimH domain through a donor strand complementation mechanism in which strand G of the N-terminal chaperone domain completes the immunoglobulin-like fold of the pilin domain (26). There is no significant contribution of the C-terminal FimC domain to FimH binding. An analogous binding mode was found for the complex between PapD and the minor P pilus subunit PapK (27).

In this paper we describe the first biochemical characterization of a bacterial pilus chaperone, using FimC as a model.
4.1.3 Experimental Procedures

4.1.3.1 Materials
DE52 and CM52 cellulose were obtained from Whatman (Maidstone, UK). Phenyl superose, PD10 and Superdex 75 HR gel filtration columns were purchased from Pharmacia (Uppsala, Sweden). Tryptone and yeast extract were from Difco (Detroit, U.S.A.). BIAcore chips were from BIAcore (Uppsala, Sweden).

4.1.3.2 Construction of expression plasmids
Molecular cloning techniques were based on Sambrook et al. (28). The gene coding for FimC (17) with its natural signal sequence and ribosomal binding site was amplified by the polymerase chain reaction (PCR) from the genome of the E. coli K12 wild type strain W3110 (29) using the primers FimC1 (5'-ATT GTT CAG CAA AGC TTC TAG AAA CAG GAC GAC AGT GAG TAA TAA-3') and FimC2 (5'-CAA AAT GAC GGG CGTAATGGA ATA AGG ATC CAA GCT TTT TTC GCC TG-3'). The gene was cloned via the XbaI and HindIII restriction sites into a derivative of pRBI-PDI-T7 (30) where fimC is under control of the T7 promoter/lac operator. The T7 terminator sequence from the vector pET11a (Stratagene, U.S.A.) was introduced at the 3' end of the fimC gene via the BamHI and HindIII restriction sites, yielding the expression plasmid pFimC. For cytoplasmic production of the C-terminal FimC domain (residues 128–205) the NdeI-BamHI fragment from pFimC-N containing the genetic sequence of the C-terminal domain without signal sequence and the T7 terminator was cloned into pRBI-PDI-T7-NdeI via NdeI and BamHI. pRBI-PDI-T7-NdeI was derived from pRBI-PDI-T7 by introduction of a NdeI site at the start codon of RBI. The plasmid pFimC-N for periplasmic expression of the N-terminal domain and cytoplasmic coexpression of the C-terminal domain was obtained by site-directed mutagenesis according to Kunkel et al. (31) using single-stranded, uridinylated DNA of pFimC, the MutageneTM Kit from BioRad (Herkules, Ca, U.S.A.) and the mutagenesis primer 5'-CCG GCT AAA TTA GCGTAACTC GAGCATGAAAGA GGGAGA CATATGGCAGAAAAATTA AGA-3'. The complete nucleotide sequences of the plasmids used in this paper has been deposited on our web site (http://www.mol.biol.ethz.ch/glockshuber/).

4.1.3.3 Expression and purification of FimC and its C-terminal domain
For production of FimC, cells of E. coli BL21(DE3) (32) transformed with pFimC were grown at 25 °C in 10 l of LB medium containing ampicillin (100 µg/ml) (LB/amp) until an
Characterization of FimC

Optical density at 600 nm (OD$_{600}$) of 1.0 was reached. After addition of IPTG (final concentration: 1 mM) the culture was grown for another 16 hours. The bacteria were harvested by centrifugation, resuspended at 4 °C in 100 ml 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mg/ml polymyxin B sulfate and stirred for 2 hours at 4 °C. After centrifugation the supernatant was dialyzed against 10 mM Tris/HCl, pH 8.0 and applied to a DE52 cellulose column. The column was washed with 10 mM Tris/HCl, pH 8.0, and the flow-through was applied to a CM52 cellulose column. FimC was eluted with a linear NaCl gradient in 10 mM Tris/HCl, pH 8.0. Fractions containing FimC were pooled, mixed with 4.0 M ammonium sulfate to a final concentration of 1.2 M ammonium sulfate and applied to a phenyl superose column. FimC was eluted with a linear gradient from 1.2 M to 0.5 M ammonium sulfate in 10 mM Tris/HCl, pH 8.0. Fractions with pure FimC were pooled, dialyzed against distilled water and stored at −20 °C.

For cytoplasmic expression of the C-terminal FimC domain, E. coli BL21(DE3) harboring pFimC-CASS was grown at 37 °C in 101 of LB/amp medium and expression was induced with 1 mM IPTG at an OD$_{600}$ of 0.5. After 3 hours the cells were harvested and subjected to periplasmic extraction as described above. The spheroplasts were disrupted in a French pressure cell (18,000 PSI) after suspension in 200 ml 50 mM Tris/HCl, pH 7.5, 0.5 mM β-mercaptoethanol, 5 mM EDTA. The inclusion bodies were washed with 1 % Triton X-100 and solubilized in 150 ml 9.0 M urea, 10 mM Mops/NaOH, pH 7.0. Insoluble material was removed by centrifugation, the supernatant was diluted to 4.0 M urea and applied to a DE52 cellulose column connected to a CM52 cellulose column in 4.0 M urea, 10 mM Mops/NaOH, pH 7.0. The flow-through containing the C-terminal domain was dialyzed against 50 mM sodium phosphate, pH 7.0, concentrated, and applied to a Superdex 75 gel filtration column. Fractions containing the pure C-terminal domain were combined, dialyzed against distilled water and lyophilized. The molecular mass of purified FimC was confirmed by MALDI mass spectrometry (calculated: 22,729.9 Da; measured 22,720.0 Da).

4.1.3.4 Protein concentration

Protein concentrations were determined by the specific absorbance at 280 nm (A$_{280}$ nm, 1 cm, 1 mg/ml) with values of 1.07 for FimC, 0.567 for the C-terminal FimC domain and 1.08 for the FimC/FimH complex (33).
4.1.3.5 Analytical gel filtration

Analytical gel filtration of FimC and its C-terminal domain was performed on a Superdex 75 HR column in 50 mM sodium phosphate, pH 7.0 at a flow rate of 10 ml/h. The FimC/FimH complex was prepared as described previously (25). Analytical gel filtration of the FimC/FimH complex was performed under the same conditions used for peptide binding studies (25 °C, PBS buffer, pH 8.0). The FimC/FimH complex was diluted with PBS to concentrations of 8.6–0.3 μM and incubated at 25 °C for at least 2 h. Samples of 50 μl were applied to a Superdex 75 HR column. Eluted proteins were detected by their absorbance at 280 nm. The apparent molecular mass of the FimC/FimH complex in PBS buffer was 42 ± 9 kDa.

4.1.3.6 Circular dichroism and fluorescence spectroscopy

Far-UV and near-UV circular dichroism (CD) spectra were recorded on a JASCO J-710 CD spectropolarimeter at protein concentrations of 1 mg/ml in 10 mM sodium phosphate, pH 7.0 at 25 °C. For far-UV CD and near-UV CD measurements, 0.2 mm and 10 mm cuvettes were used, respectively. All fluorescence measurements were performed on a HITACHI F 4500 fluorescence spectrometer at 25 °C.

4.1.3.7 Urea-induced unfolding equilibria and stopped-flow measurements

Unfolding/refolding equilibria of FimC and its C-terminal domain were measured at 25 °C and a constant ionic strength of 92 mM (calculated according to 34). The following buffers containing different concentrations of urea were used: pH 7.0: 50 mM sodium phosphate; pH 4.0: 50 mM formic acid/NaOH, 57 mM sodium chloride; pH 2.0: 50 mM sodium phosphate, 68 mM sodium chloride. The native or unfolded proteins (in 6.0 M urea) were diluted with the above buffers to a final protein concentration of 1.0 μM in the case of FimC and to a concentration of 2.8 μM in the case of the C-terminal domain, and incubated at 25 °C for 3 days. Equilibrium transitions were followed by the protein fluorescence at 335 nm (excitation at 295 nm) in the case of FimC and at 302 nm (excitation at 280 nm) in the case of the C-terminal domain. Data were evaluated according to the two-state model of folding (35) using a six-parameter fit (36) and normalized. The urea-dependence of the apparent rate constant of unfolding and refolding of the C-terminal domain were measured fluorimetrically with a SX-17MV stopped-flow reaction analyzer (Applied Photophysics, Leatherhead, UK) at 25 °C in 50 mM sodium phosphate, pH 7.0. The native or urea-denatured protein (in 3.0 M urea) was diluted 1:11 to a final protein concentration of 5.0 μM with 50 mM sodium phosphate, pH 7.0.
Characterization of FimC containing different concentrations of urea. The fluorescence intensity above 305 nm (excitation at 280 nm) was recorded for 0.5 s and the fluorescence traces were analyzed according to a single first-order reaction. The urea-dependence of the averaged apparent first order rate constants was evaluated according to the equation

\[
\ln k_{app} = \ln [k_f^{H_2O} \exp (-m_f [\text{urea}]) + k_u^{H_2O} \exp (m_u [\text{urea}])],
\]

where \(k_{app}\) is the apparent rate constant of folding/unfolding, \(k_f^{H_2O}\) and \(k_u^{H_2O}\) are the rate constants of folding and unfolding in the absence of denaturant, respectively, \(m_f\) and \(m_u\) give the linear dependence of \(\ln k_f\) and \(\ln k_u\) on the denaturant concentration and [urea] is the concentration of urea (37).

4.1.3.8 Peptide binding measurements

Peptide binding of FimC was investigated with a synthetic peptide consisting of the C-terminal 11 residues of the pilus subunit FimH extended by a hydrophilic N-terminal tetrapeptide (S-R-R-S) (overall sequence: H3N+-S-R-R-S-Q-S-I-I-G-V-T-F-V-Y-Q-C02'). The peptide was biotinylated at its free N-terminus by mixing with D-biotinyl-e-aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim, Germany) at a 1:1 ratio (concentrations of 0.2 mM each) in 50 mM sodium phosphate, pH 8.0, 100 mM NaCl, 0.1 mM EDTA and incubation for 1 h at room temperature. Peptide binding experiments were performed on a BIAcore surface plasmon resonance instrument (Pharmacia Biosensor AB) at 25 °C in PBS buffer using a microsensor chip covalently coated with streptavidin. 10 μl of a 20 μM solution of the biotinylated peptide were injected at a flow rate of 5 μl/min to allow binding of the peptide to the sensor chip. After washing with PBS, the chip was equilibrated with 10 μl of a solution of FimC (1–100 μM) in PBS, then washed with PBS and the amplitude of the first-order signal decrease during recovery of the baseline was recorded for all FimC concentrations after washing for 10 s. The apparent dissociation constant (K_d^app) of the FimC/peptide complex was deduced by fitting the data according to the equation

\[
S = S_p/(1+ [\text{FimC}]/ K_d^{app}) + S_b (1- 1/(1+ K_d^{app} /[\text{FimC}]),
\]

where \(S\) is the resonance signal after washing for 10 s, \(S_p\) is the signal in the absence of FimC, \(S_b\) is the signal after washing for 10 s when all binding sites are occupied by FimC before washing and [FimC] is the concentration of FimC in solution prior to washing. It was verified that no direct binding of FimC to streptavidin occurred.
4.1.4 Acknowledgements

We thank Gerhard Frank for N-terminal sequencing and Peter James for recording mass spectra.
4.1.5 Results

4.1.5.1 Expression and purification of FimC and its C-terminal domain.

FimC was overproduced in *E. coli* and purified to homogeneity by conventional chromatography with yields of 30 mg FimC per liter of bacterial culture (Figure 2A). To study the influence of domain interactions on the stability of FimC in vitro, we intensively tried also to produce milligram quantities of the isolated N-terminal domain (residues 1-121; pFimC-N), the N-terminal domain extended by the linker peptide (residues 1-132; pFimC-N-L) and the isolated C-terminal domain (residues 128-205; pFimC-C) (Figure 1) under control of the T7 promoter/lac operator system in the periplasm of *E. coli* BL21(DE3). However, none of the domains could be detected in the periplasmic or insoluble fractions at growth temperatures between 25 and 37 °C (data not shown). To overcome this problem, we removed the bacterial signal sequences and tried to express the isolated FimC domains in the cytoplasm of BL21(DE3). Only the isolated C-terminal domain (FimC-C) could be obtained in reasonable amounts and was purified from inclusion bodies with a yield of 15 mg of pure FimC-C per liter of bacterial culture (Figure 2A). In contrast, cytoplasmic expression of the N-terminal domain constructs again could not even be detected after immunoblotting (data not shown). Limited proteolysis of FimC using subtilisin, proteinase K, thrombin and trypsin also did not yield fragments corresponding to the N-terminal domain of FimC (data not shown). Analysis of FimC and the isolated C-terminal FimC domain by analytical gel filtration experiments revealed molecular masses of 27 (± 5) kDa for FimC and 8 (± 5) kDa for FimC-C, corresponding to the monomeric states of both proteins (Figure 2B).

4.1.5.2 Folding and thermodynamic stability of FimC and its isolated C-terminal domain.

For further characterization of the C-terminal domain, its spectroscopic properties were compared with those of wild type FimC. As expected for a β-sheet protein, the far-UV circular dichroism (CD) spectrum of FimC shows a minimum at 217 nm (molar mean residue ellipticity -5300 deg cm² dmol⁻¹) (Figure 3A). However, the spectrum exhibits an unusual second minimum at 195 nm. This is almost certainly due to the intrinsic spectroscopic properties of the C-terminal domain, which lacks the β-sheet-specific minimum around 215 nm and displays a strong minimum at 196 nm (Figure 3A). The presence of a defined tertiary structure in the isolated C-terminal domain is however evident from a characteristic
Characterization of FimC

fine structure with positive ellipticity in its near-UV CD spectrum, whereas the near-UV CD spectrum of FimC shows negative ellipticity (Figure 3B). Both tryptophan residues of FimC (Trp36 and Trp84) are located in the N-terminal domain (21; 25). In accordance with the NMR structure of FimC, its fluorescence maximum at 345 nm indicates that the tryptophans are partially solvent exposed. As expected, the isolated C-terminal domain exhibits a pure tyrosine fluorescence spectrum (maximum at 304 nm) (Figure 3C). The fluorescence intensity of both FimC and its C-terminal domain decreases significantly after unfolding of the proteins by 6.0 M urea (Figure 3C). Urea-induced unfolding of FimC at pH 7.0 and 4.0, followed by fluorescence, yielded cooperative one-step transitions that were fully reversible (Figure 4). Evaluation of the data according to the two-state model of folding yielded free energies of folding of $-38.2$ and $-32.7 \text{ kJ/mol}$, respectively (Table I). The C-terminal FimC domain proved to be strongly destabilized compared to FimC wild type. The domain is only marginally stable with a free energy of folding of $-4.2 \text{ kJ/mol}$ at pH 7.0 (Figure 5A, Table I). The apparent rate constants of unfolding and refolding of the C-terminal FimC domain at pH 7.0 and different urea concentrations, measured with stopped-flow tyrosine fluorescence, were consistent with two-state folding and reproduced the equilibrium measurements within experimental error (Figure 5B, Table I). The C-terminal FimC domain lacks cis-prolines (21) and the fluorescence traces obtained for the unfolding and refolding reactions could be described by single exponential functions at all urea concentrations. In addition, no rapid burst phases within the dead time of the stopped-flow measurement (2 ms) were observed, indicating the absence of a hydrophobic collapse prior to tertiary structure formation of the C-terminal domain. The slopes of the folding and unfolding branches in Figure 5B showed that the transition state of folding of the C-terminal domain is closer to the native than to the unfolded state ($\alpha = 0.68$). Extrapolation of the folding and unfolding branches in Figure 5B to 0 M urea yields $15 \text{ s}^{-1}$ for the rate constant of folding and $3.4 \text{ s}^{-1}$ for the rate constant of unfolding.

4.1.5.3 FimC only weakly binds to the C-terminal peptide of the type 1 pilus adhesin FimH.

To study the affinity of FimC to the C-terminal segments of type 1 pilus subunits, we focused on the C-terminal peptide of the type 1 pilus adhesin FimH for a comparison with the extraordinarily stable complex between FimC and intact FimH (25; 26). For this purpose, a synthetic peptide corresponding to the C-terminal 11 residues of FimH, extended by the tetrapeptide Ser-Arg-Arg-Ser at the N-terminus to increase solubility was used. The peptide
was then biotinylated at the free N-terminus and coupled to a BIAcore sensor chip coated with streptavidin. The chip was purged with buffer containing different concentrations of FimC, and the amount of bound FimC at the different FimC concentrations was determined by surface plasmon resonance (Figure 6). The binding data at pH 8.0 and 25°C were consistent with a single chemical binding equilibrium, yielding an apparent dissociation constant ($K_D$) of $8 \pm 1 \mu M$ (Figure 6). The stability of the intact FimC/FimH complex against dissociation was investigated under the same conditions by analytical gel filtration at the lowest concentration of the complex that still could be detected by absorbance spectroscopy. No significant dissociation could be detected at concentrations as low as 0.3 $\mu M$ in the applied sample and 20 nM in the elute (data not shown). Consequently, the $K_D$ of the complex must be below 10 nM. Binding of the C-terminal FimH peptide to FimC is thus at least 1000-fold weaker compared to binding of intact FimH.
4.1.6 Discussion

The assembly factor FimC has been identified as an essential component for the formation of adhesive type 1 pili (16; 17). As a prerequisite for future studies on the in vitro reconstitution of type 1 pili in the presence of FimC, we describe here the biochemical and biophysical properties of FimC and the isolated C-terminal domain. Several lines of evidence indicate that the interactions between both immunoglobulin-like domains of FimC are essential for the overall thermodynamic stability of the chaperone and its stability against degradation in vivo. While the isolated N-terminal domain appears to be so unstable that it can not be overexpressed in E. coli, the isolated C-terminal domain could be purified, and its stability was compared with that of FimC. Although the C-terminal FimC domain is an autonomous folding unit, it proved to be an extremely unstable protein (ΔG = −4.2 kJ mol⁻¹) with a significant fraction of the domain already unfolded even in the absence of denaturant. In contrast, intact FimC is an about 10-fold more stable protein, with a free energy of folding of −38.2 kJ mol⁻¹ at pH 7.0. FimC is thus composed of two comparably unstable modules, and the inter-domain contacts are the most important interactions that contribute to its thermodynamic stability. The importance of the inter-domain interactions is also evident from a well-defined hydrophobic core between both domains and the highly conserved salt bridge formed by the residues Glu80, Arg116 and Asp192 in the solution structure of FimC (27).

The two-domain architecture of periplasmic pilus chaperones could have been a special advantage during the evolution of these highly specific binding proteins. Destabilizing mutations in individual chaperone domains occurring during evolution towards their actual function could have been compensated by mutations that strengthened the interaction between the domains. An analogous example in this context is γB crystallin from the vertebrate eye lens. This protein also mainly recruits its enormous stability from inter-domain interactions. γB crystallin evolved by gene duplication and fusion of two homologous β-sheet domains, but the isolated domains are much less stable than the intact protein (38; 39).

Recently, the interactions between FimC and FimH have been characterized by TROSY-NMR in solution (25) and X-ray crystallography (26). In contrast to previous studies on complexes between PapD and synthetic peptides corresponding to the C-terminal residues of P-pilus subunits (19; 22), it was found that the surface area of FimC covered by FimH is much larger, and that FimC very specifically interacts with the folded form of the adhesin. In the X-ray structure of the FimC/FimH complex the adhesin FimH is folded into two domains, the N-terminal lectin domain and the C-terminal pilin domain which is supposed to anchor the
Characterization of FimC

adhesin domain to the pilus. In the FimC/FimH complex, only the pilin domain of FimH interacts with FimC. The contact between the C-terminal β-strand of the pilin domain and FimC involves β-sheet hydrogen bonding to the G1 strand of FimC, which completes a lacking β-strand in the immunoglobulin-like pilin fold (26). The low affinity (8 μM) of FimC for the C-terminal FimH peptide, and the at least 1000-fold higher affinity toward the intact, folded FimH found in this study are in good agreement with the structural data which revealed multiple FimC/FimH contacts in addition to binding of the C-terminal FimH β-strand (25; 26; 27). Our gel filtration experiments showed that the purified complex does not dissociate when it is applied at concentrations of 0.3 μM and eluted at concentrations of 20 nM. This is in line with a previous study on the binding of the FimC/FimH complex to FimD, where no dissociation of the complex at concentrations as low as 75 nM was observed (20). The low affinity of FimC for the C-terminal FimH peptide also has implications for the folding of individual pilus subunits in that it questions that FimC already interacts with unfolded pilus subunits prior to subunit folding, as suggested by Soto et al. (20). FimC rather appears to maintain folded subunits in a translocation- and assembly-competent state in the periplasm, and prevent premature association of folded subunits in the periplasm (40). On the other hand, however, the strong interaction between FimC and intact, folded FimH raises the question of how an efficient dissociation of the FimC/FimH complex is accomplished in vivo when FimC delivers the adhesin to the assembly platform FimD in the outer bacterial membrane. As the assembly of a complete pilus in E. coli occurs within 1-3 minutes (3), it seems that the FimC/FimH complex is less stable in vivo. The most likely mechanism for an induced dissociation of the complex seems to be a conformational change in the complex triggered by a specific contact with FimD.
Figure 1. Ribbon drawing of the NMR structure of FimC. The N-terminal domain (residues 1–115) and the C-terminal domain (residue 131–205) are colored in red and blue, respectively. The domains are connected by a 15-residue linker peptide shown in gray that contains a small 3$_{10}$-helix (segment 126–130). The positions of the C-terminal residues of the FimC segments encoded by the plasmids pFimC-N and pFimC-N-L (A121 and R132, respectively), and the first amino acid of the cytoplasmically expressed C-terminal domain (A128) are indicated by arrows. The figure was generated with the program MOLMOL (44).
Figure 2. FimC and its isolated C-terminal domain (residues 128-205) are monomers. (A) Purification of the C-terminal FimC domain from cytoplasmic inclusion bodies. A reducing 17 % (w/v) polyacrylamide-SDS gel stained with Coomassie Blue is shown. Lane 1, molecular mass standard; lane 2, total cell extract of induced cells of E. coli BL21(DE3)/pFimC-CΔSS; lane 3, insoluble fraction of the cell extract; lane 4, pooled fractions after solubilization and chromatography on DE52 cellulose and CM52 cellulose in the presence of 4.0 M urea; lane 5, purified C-terminal domain after refolding and gel filtration on Superdex 75 under native conditions; lane 6, purified FimC. (B) Analytical gel filtration of FimC and its C-terminal domain on a Superdex 75 column (30 x 1 cm) in 50 mM sodium phosphate (pH 7.0) at 22 °C. FimC (solid line) and the C-terminal domain (dashed line) were applied at concentrations of 20 μM and 100 μM, respectively. The dotted line corresponds to a mixture of proteins used as molecular mass standards. Proteins were detected by their absorbance at 280 nm.
Figure 3. Spectroscopic characterization of FimC and its C-terminal domain. (A) Far-UV and (B) near-UV circular dichroism spectra of FimC (solid lines) and its C-terminal domain (dotted lines) at 25 °C in 10 mM sodium phosphate, pH 7.0. (C) Fluorescence emission spectra of FimC and its C-terminal domain. (1) native FimC, (2) unfolded FimC in 6.0 M urea (excitation at 295 nm), (3) native C-terminal domain, (4) unfolded C-terminal domain in 6.0 M urea (excitation at 280 nm). Protein concentrations were 1 μM for FimC and 2.8 μM for the C-terminal domain.
Figure 4. Urea-dependent unfolding/refolding equilibria of FimC at pH 4.0 (squares) and at pH 7.0 (triangles) at 25 °C, followed by fluorescence emission at 335 nm (excitation at 295 nm). Unfolding and refolding experiments are represented by open and closed symbols, respectively. The original fluorescence data were fitted according to the two-state-model and normalized (pH 7.0, solid line; pH 4.0, dashed line).
Figure 5. Urea-dependent unfolding/refolding equilibrium of the C-terminal FimC domain at pH 7.0 at 25 °C, measured by tyrosine fluorescence (excitation at 280 nm). (A) Equilibrium unfolding transition. The solid line corresponds to a two-state analysis. (B) Urea-dependence of the apparent rate constant (k_{app}) of unfolding and refolding of the C-terminal domain measured by stopped-flow fluorescence. The urea-dependence of k_{app} was fitted according to the two-state model (solid line).
Figure 6. Determination of the apparent dissociation constant ($K_{D}^{app}$) of the complex between FimC and the peptide $H_{2}N^{\text{-}}$S-R-R-S-Q-S-I-I-G-V-T-F-V-Y-Q-CO$_{2}$ containing the 11 C-terminal residues of FimH (italics). The peptide was biotinylated at its N-terminus and immobilized on a BIAcore sensor chip coated with streptavidin. The binding of FimC to the peptide was measured at pH 8.0 and 25 °C. The chip was equilibrated with a solution of FimC (1–100 μM), then washed with buffer and the amplitude of the signal change during recovery of the baseline (given in resonance units (RU)) after 10 s of washing was determined. The solid line corresponds to a fit according to a single chemical binding equilibrium.
**Table I.** Thermodynamic stabilities of FimC and its C-terminal domain (FimC-C) at 25°C deduced from urea-dependent equilibrium transitions and stopped-flow fluorescence measurements

<table>
<thead>
<tr>
<th></th>
<th>ΔG [kJ mol⁻¹]</th>
<th>Cooperativity (m-value)</th>
<th>Midpoint of transition [kJ mol⁻¹ M⁻¹]</th>
<th>[M urea]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FimC, pH 7.0</td>
<td>-38.2 ± 1.3</td>
<td>12.8 ± 0.4</td>
<td>3.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>FimC, pH 4.0</td>
<td>-32.7 ± 1.8</td>
<td>11.4 ± 0.6</td>
<td>2.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>FimC-C, pH 7.0</td>
<td>-4.2 ± 0.9</td>
<td>4.6 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>FimC-C, pH 7.0,</td>
<td>-3.7 ± 0.3</td>
<td>5.3 ± 0.5</td>
<td>0.7 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

stopped flow kinetics

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a Fitting the data in Figure 5B according to the two-state model yields values of 15.1 ± 1.1 s⁻¹ and 3.4 ± 0.2 s⁻¹ for the rate constants of folding (kfH2O) and unfolding (kH2O) in the absence of urea, respectively. The slopes of the linear dependence of the logarithm of kf and ku on urea concentration (Figure 5B) have the following values: m\(f\) = -1.45 ± 0.2 M⁻¹; m\(u\) = 0.68 ± 0.02 M⁻¹. The equation m = (m\(u\) - m\(f\)) RT yields the calculated m-value for the equilibrium transition (5.3 ± 0.5 kJ mol⁻¹ M⁻¹), which agrees within experimental error with the equilibrium measurement (4.6 ± 0.5 kJ mol⁻¹ M⁻¹). The \(\alpha\)-value of 0.68, calculated from \(\alpha = |m\(f\)| / (|m\(f\)| + |m\(u\)|), indicates that the transition state of folding is closer to the native than to the unfolded state.
4.1.7 References


4.2 Chaperone-controlled stoichiometry of a macromolecular assembly*

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4.2.1 Summary

The assembly of adhesive type 1 pili in *Escherichia coli* depends on the periplasmic chaperone FimC. We have randomized 9 distinct residues in the subunit binding site of FimC and tested 1400 FimC variants for their ability of complementing FimC deficiency in a *fimC* null strain. More than 600 FimC variants were sequenced and 116 variants were characterized in detail with respect to recovery of adhesiveness and pilus phenotype. Most of the FimC variants dramatically affected the number of pili per cell, as well as the length and subunit composition of the pili. We conclude that bacterial pilus chaperones have fine-tuned affinities for the different pilus subunits, and that these affinities regulate both the initiation of pilus assembly and pilus stoichiometry.
4.2.2 Introduction

The first step in the infection of hosts by pathogenic Gram-negative bacteria is the bacterial attachment to target tissues. This process is mediated by filamentous surface organelles termed pili or fimbriae. Pili are large, heterooligomeric protein complexes which contain adhesin subunits that specifically recognize receptor moieties on the surface of mammalian cells (Hultgren et al., 1993; Klemm and Krogfelt, 1994). An important member of these adhesin-presenting fibers are type 1 pili, which are found on more than 85% of all isolated *Escherichia coli* strains and are required for all known urinary tract infections by *E. coli* (Klemm and Krogfelt, 1994). Type 1 pilus filaments are anchored to the outer *E. coli* membrane and consist of 500–2000 protein subunits, which are arranged in a right-handed helix (Brinton, 1965; Klemm and Krogfelt, 1994). A single *E. coli* cell may contain 500 to 700 type 1 pili on its surface that are generally 0.5–2 µm long and 7 nm wide (Brinton, 1965). Nine genes are required for effective type 1 pilus biogenesis, which are clustered at 98 minutes of the *E. coli* K-12 chromosome (Blattner et al., 1997). FimA, the main structural subunit, comprises about 98% of subunits of the pilus, while the remaining 2% are three minor subunits FimF, FimG and FimH. FimH represents the type 1 pilus adhesin and is found at the tip of the pilus (Jones et al., 1995). FimH is a critical virulence factor of uropathogenic *E. coli* strains and specifically binds to mannose-containing receptors on target cells. Additionally, type 1 pili mediate the survival of pathogenic *E. coli* cells inside of marophages and may thus be responsible for recurrent infections of the urinary tract (Baorto et al., 1997).

Type 1 pili belong to the group of bacterial adhesive organelles, which are assembled via the so-called chaperone/usher pathway. This pathway requires two specific protein factors. i) the “usher” or assembly platform protein FimD, which mediates translocation of pilus subunits through the outer membrane and anchors the pilus to the bacterial surface (Klemm and Krogfelt, 1994), and ii) the soluble periplasmic chaperone FimC which binds to the individual type 1 pilus subunits in the periplasm and specifically delivers the subunits to FimD (Saulino et al., 1998).

The three-dimensional structure of the 23 kDa assembly factor FimC, solved by NMR, consists of two globular domains, each possessing an immunoglobulin-like fold (Figure 1). The orientation of the domains relative to each other is defined by an invariant salt bridge in the inter-domain cleft and a well-defined hydrophobic core in the hinge region between the domains (Pellecchia et al., 1998). The NMR structure of FimC is very similar to the crystal
structure of the related P pilus chaperone PapD (Holmgren and Brändèn, 1989), which shares
34% sequence identity with FimC.

The crystal structure of PapD in complex with a synthetic peptide corresponding to the C-terminal segment of the P pilus adhesin PapG suggested that pilus chaperones recognize the subunits by binding their strongly conserved C-terminal decapptide regions via main chain hydrogen bonds in a β-sheet-like manner (Kuehn et al., 1993; Soto et al., 1998). Subsequent analysis of the stable complex between the intact FimH adhesin and FimC with transverse relaxation-optimized (TROSY) NMR spectroscopy (Pervushin et al., 1997) however, revealed that the binding area of FimC for the adhesin is much larger than that found in the PapD peptide complex. Moreover, numerous FimC residues outside of the surface area corresponding to the peptide binding site of PapD are involved in the interaction with FimH. The mapped FimC binding surface represents a patchwork of charged, polar and hydrophobic side chains (Pellecchia et al., 1999). This, together with the observation that FimC binds with an at least 1000-fold lower affinity to a peptide corresponding to the 11 C-terminal FimH residues (Hermanns et al., submitted) indicated that FimC specifically recognizes the tertiary structure of the adhesin (Pellecchia et al., 1999). This is in contrast to most other chaperones that unspecifically bind unfolded or partially folded polypeptides and that inhibit protein aggregation during folding (Hartl, 1996; Beissinger and Buchner, 1998).

The NMR data were entirely confirmed by the recent X-ray structure determinations of the FimC/FimH complex (Choudhury et al., 1999) and the complex between PapD and P pilus subunit PapK (Sauer et al., 1999). Both structures revealed that the pilus subunits form domains with an immunoglobuline-like fold similar to the N-terminal domains of FimC and PapD, but lack a β-strand which is complemented by the strand G1 from the N-terminal chaperone domains in the respective complexes. As all P and type 1 pilus subunits share significant sequence identity, the structure of FimH and PapK also allowed modeling of the helical assembly of subunits in both pilus systems, assuming a similar donor strand complementation mechanism at the subunit/subunit contact level. In addition to its pilin domain, FimH contains an additional N-terminal mannose binding domain does not interact with the chaperone in the FimC/FimH complex (Choudhury et al., 1999).

To investigate the molecular mechanism of subunit binding by FimC and the contribution of chaperone surface residues in more detail, we have performed an extensive random mutagenesis study on the function of nine residues in the subunit binding site of FimC. We constructed five plasmid libraries of FimC variants with amino acid replacements in one or two of these nine residues. The libraries were used to transform an E. coli fimC null mutant.
1400 of the resulting clones were tested for complementation of FimC deficiency using bacterial agglutination with yeast cells as sensitive phenotypic criterion. More than 600 complementing and non-complementing FimC variants were sequenced, and 116 of them were characterized in detail with respect to FimC expression levels and analysis of the number and length of the resulting type 1 pili with electron microscopy. In addition, the isolated FimC domains and FimC variants with amino acid exchanges of residues that form the interdomain salt bridge R116-D192 in wild type FimC were tested for complementation of FimC deficiency in W3110ΔfimC.

We show that amino acid replacements at the subunit binding site of FimC do not only affect the pilus length and pilus number per cell, but also the subunit composition of the pili. Our data clearly demonstrate a fine-tuned affinity of pilus chaperones for the individual pilus subunits that determines both initiation of pilus assembly and pilus stoichiometry.
4.2.3 Experimental Procedures

4.2.3.1 Reagents

Synthetic oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany) and isopropyl-β-D-thiogalactoside (IPTG) was from AGS GmbH (Heidelberg, Germany). Tryptone and yeast extract were from Difco (Detroit, U.S.A.). Polyclonal anti-FimC and anti-FimH rabbit antibodies were purchased from Dr. Max Rosskopf (Zürich, Switzerland), polyclonal anti-FimA rabbit antibodies were from Eurogentec (Herstal, Belgium). Alkaline phosphatase-conjugated goat anti-rabbit antibodies were obtained from Dianova (La Roche, Switzerland) and chromogenic substrates for Western blots were from Biomol (Hamburg, Germany). Dry yeast and low fat milk powder were from MIGROS (Switzerland). All other chemicals were from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) or Sigma-Aldrich (Deisenhofen, Germany) and of the highest purity available.

4.2.3.2 General methods

Molecular cloning techniques were based on Sambrook et al., (1989). Plasmid DNA was prepared by using the Promega Plasmid-Miniprep Spin Kit according to the manufacturer’s protocol. DNA sequencing was performed on a LICOR DNA 4000 Lab Sequencer (MWG-BIOTECH) using the VISTRA system sequencing kit (Amersham). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Fling and Gregerson, (1986) with 15% (w/v) acrylamide gels.

4.2.3.3 Random mutagenesis on different FimC residues and construction of expression plasmids

Random mutagenesis of fimC was performed according to Kunkel et al., (1987), using the Mutagene TM Kit from BioRad (Herkules, CA, U.S.A.) and uridinylated, single-stranded DNA of the expression plasmid pFimC (Hermanns et al., submitted) as template. To avoid any background of active wild type FimC after random mutagenesis of residues T7/R8, E80, W84/N86, L103/A106, R110/K112, we first constructed five fimC genes with stop codons at the residues to be randomized with the following oligonucleotide primers:

FimCT7R8ko: 5’-CCC TGC CGG ATA GAT AGG CCT ATT AGC CTA GGG CAA CAC CCG CTT CAG C-3’; FimCE80ko: 5’-GCT TTA ACG TTC ATC CAG AAC AAA GGC CTA TTA CGG CAG CTG GTT ATT TGT TG-3’; FimCW84N86ko: 5’-CCA TTG ACG GAA TCG CTT CAG C-3’; FimCL103A106ko:
5'-GCC GGG CGA TAG TAA AGC TTA ATG CGG GAG ATA GGC CTA TTA ATT CTC GGT GAG TTT TGA TTT ATC-3'; FimCR110K112ko: 5'-CTA ATT TAG CCG GAC GGT AGT AAG GCC TAT TAG ATA ATC GCG AGC TGT AGC G-3'.

The five resulting FimC deficient plasmids were then used as templates for the random mutagenesis which was performed with the following primers:

FimCT7R8: 5'-CCC TGC CGG ATA GAT TAC NNN NNN CGC GCC TAG GGC AAC ACC CGC-3'; FimCE80: 5'-GCT TTA ACG TTC ATC CAG AAT AAG GANNNC CGG TCT TGG GC AGC TGG TTA TTT GTT G-3'; FimCW84N86: 5'-CCA TTG ACG GAA TCA TGTA TAA CNN NCA TNN NGA ATA AGC TTT CCC GGT CC-3'; FimCL103A106: 5'-GTA AAG CTT AAT GCG GGA GAT AAT NNN GAG CTG NNN CGT ATT CTC GGT GAG TTT TGA TTT ATC-3'; FimCR110K112: 5'-GCC GGA CGGTAG TAGAGNNNAATTNNNGCTGATAATC GCGAGCTG-3'.

Plasmids for periplasmic expression of the isolated C-terminal FimC domain and the FimC variants R116D, D192R and R116D/D192R were constructed by site-directed mutagenesis of pFimC with following primers: isolated C-terminal domain (residues 128–205) (pFimC–C): 5'-CGG ACG CGC TGA AGC GGC AGA AAA ATT AAG A-3'; replacement of R116D (pFimC–R116D): 5'-CAT TAA ACT GT A CT TA AGC TCC GGC TAA GCT TGC GTT GCC ACC CGA TC-3'; replacement D192R (pFimC–D192R): 5'-TAC CGA ACA ATA AAT CGA TAT GCC GCA CT T ACC-3'. The plasmid for periplasmic expression of the N-terminal FimC domain (residues 1–121) (pFimC–N) was described previously (Hermanns et al., submitted).

4.2.3.4 Construction of the strain W3110ΔfimC

The selective disruption of the fimC gene in the genome of E. coli W3110 (Bachmann, 1972) was performed by allelic exchange (Hamilton et al., 1989). First, we deleted a 466 bp fragment in pFimC (corresponding to bases 121–586 in the fimC gene) by digestion with Bsu36I and SmaI, filling in the protruding ends and religating the vector. The disrupted gene (fimCA121–586) was cloned into the vector pMAK705 (Hamilton et al., 1989) via XbaI and HindIII. E. coli W3110 was transformed with the resulting plasmid pMAK705–fimCA121–586. After selection for incorporation of the plasmid into the genome, individual clones were screened for allelic exchange and subsequent loss of the plasmid by their inability to agglutinate with yeast cells. The resulting fimC null mutant, termed W3110ΔfimC, was verified by amplification of the shortened fimC gene from the genome by PCR, and the recovery of agglutination after transformation with pFimC.
4.2.3.5 Screening for active and inactive FimC variants by agglutination with yeast cells

The plasmid DNA preparations of the five different fimC libraries were first digested with StuI to remove residual template DNA (all mutagenesis primers that introduced the stop codon in the templates also introduced a single StuI site). *E. coli* W3110ΔfimC was then transformed with the intact plasmids. The resulting clones were tested for their ability to agglutinate with yeast cells. Briefly, 0.5 ml of LB medium containing ampicillin (100 μg/ml) and IPTG (1 mM) were inoculated with single colonies and incubated at 37 °C for 16 h without shaking. Bacteria were then mixed with 50 μl of 10% (w/v) dry bakers yeast in 10 mM Na2HPO4, 4 mM KH2PO4, 150 mM NaCl (PBS buffer) (Sauter, 1992). The extent of agglutination after 10 min was examined and classified into five different categories.

4.2.3.6 Detection of periplasmic FimC concentrations by immunoblotting

Static cultures of W3110ΔfimC harboring plasmids for expression of different FimC variants were grown in 10 ml LB/amp/IPTG medium at 37 °C for 24 h. Portions of 200 μl were used to inoculate 10 ml of the same medium and static cultures were grown for 24 h. Bacteria were harvested by centrifugation, suspended at 4 °C to a final OD550 nm of 100 in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mg/ml polymyxin B sulfate and incubated for 2 h at 4 °C. After centrifugation, the supernatants (periplasmic extract) were subjected to SDS-PAGE (15%) and the separated proteins were blotted onto a PVDF membrane. For immunospecific staining of FimC, polyclonal anti-FimC rabbit antibodies were used (Blake et al., 1984). Periplasmic extracts of W3110ΔfimC and W3110ΔfimC harboring pFimC were analyzed as controls.

4.2.3.7 Electron microscopy

Static cultures of the *E. coli* strains W3110, W3110ΔfimC and W3110ΔfimC harboring expression plasmids for the various FimC variants were grown for 24 h at 37 °C in LB or LB/amp/IPTG medium, diluted 1:20 with the same medium and again grown for 24 h. After centrifugation at 2500 x g cells were suspended in 320 mM Tris/acetate, pH 7.5, 8 mM EDTA (TAE) and again centrifuged. Bacteria were suspended in distilled H2O and adsorbed for 2 min to 7 nm thick glow discharged carbon support films mounted on 300-mesh per inch copper grids. Samples were then either air-dried and rotary shadowed with 2 nm platinum-carbon (95/5) at an elevation angle of 10°, or negatively stained with 1% Na-phosphotungstate, pH 7.4, for 35 s.
For production of polyclonal rabbit antibodies against the different pilus subunits FimH was overexpressed and purified as described (Pellecchia et al., 1999). FimA was purified from type 1 pilus preparations. For this purpose, the fim deficient E. coli strain AAEC189 (Blomfield et al., 1991) was transformed with the plasmid pSH2 containing the complete fim gene cluster (Orndorff and Falkow, 1984). The cells were grown at 37 °C for 16 h in 101 of LB medium containing chloramphenicol (100 μg/ml). The bacteria were harvested by centrifugation, resuspended in 120 ml PBS and the pili were shared off mechanically. After centrifugation at 2500 x g type 1 pili were precipitated by addition of 1 M Mg₂Cl₂ to the supernatant (final concentration: 0.1 M) and incubation for 1 h at room temperature. After centrifugation pili were solubilized in PBS and the pili were dissociated by incubation with 6 M GdmCl for 2 days. After dialysis against 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, FimA (15 kDa) was separated from FimH (29 kDa) by preparative gel filtration on a Superdex 16/60 column in the same buffer.

Colloidal gold-antibody complexes were prepared as described (Slot and Geuze, 1985; Baschong and Wrigley, 1990). For immunospecific labeling of the subunits, the bacterial cultures were centrifuged at 2500 x g, suspended in TAE, again centrifuged and resuspended in 100 mM Tris/HCl, pH 7.4, 1% (w/v) BSA. Bacteria were incubated for 1 h at room temperature, adsorbed for 2 min to 7 nm thick glow discharged carbon support films mounted on 300-mesh per inch gilded nickel grids. The bacteria were then incubated for 45 min on 50 μl droplets of 100 mM Tris/HCl, pH 7.5, 0.1% (w/v) BSA either containing gold coupled anti-FimH antibodies (4.6 ng/μl) or gold-labeled anti-FimA antibodies (70 ng/μl). Bacteria were washed with decreasing concentrations of Tris/HCl, pH 7.5 and negatively stained with 1% Na-phosphotungstate, pH 7.4, for 30 s. Bacteria were examined in a Philips CM12 electron microscope (Eindhoven, The Netherlands) at an acceleration voltage of 100 kV. Images were recorded with a Gatan multiscan CCD camera (Gatan Inc. Pleasanton, CA).
4.2.4 Acknowledgements

We thank Jens Hennecke for his help during construction of the expression plasmids, Gerhard Frank for N-terminal sequencing and Peter James for recording mass spectra.
4.2.5 Results and Discussion

The recently solved X-ray structure of the FimC/FimH complex describes the exact binding mechanism between the C-terminal pilin domain of FimH and FimC (Choudhury et al., 1999). The structure shows that the final C-terminal β-strand of FimH forms a parallel β-strand interaction with the G1 strand of FimC that completes the β-barrel structure of the FimH pilin domain.

To gain insight into the substrate specificity of FimC which is supposed to interact with the homologous type 1 pilus subunits FimA, FimF and FimG in a similar manner, nine FimC residues in the subunit binding site were randomized (Figure 1). The nine selected amino acids belong to the consensus sequence of conserved residues in the FimC-PapD chaperone family and are present in at least 70% of all known periplasmic pilus chaperones (Hung et al., 1996; Bond et al., 1997). Additionally, with exception of E80, all are surface-exposed residues from the N-terminal FimC domain that were found to interact with FimH both by TROSY-NMR (Pellecchia et al., 1999) and X-ray crystallography (Choudhury et al., 1999).

4.2.5.1 Random mutagenesis of nine distinct FimC residues

In one fimC library T7 and R8 were randomized in parallel. Both residues are located in β-strand A1” of FimC (Pellecchia et al., 1998). In the crystal structure of FimH in complex with FimC the chaperone residue R8 forms a hydrogen bond to the free carboxy-terminus of the adhesin which anchors FimH to the bottom of the chaperone cleft (Choudhury et al., 1999). In the crystal structure of the P pilus chaperone PapD with a peptide corresponding to the 19 C-terminal amino acids of the subunit PapG, the chaperone residue T7 was also found to make van der Waals contact to the side chain of the C-terminal residue in the peptide (Kuehn et al., 1993).

E80 was selected because it is involved in the interdomain salt bridge together with R116 and D192 which is highly conserved among the members of pilus chaperones and contributes to the relative orientation of the N- and C-terminal domain of FimC (Pellecchia et al., 1998).

The two randomized amino acids W84 and N86 are located in β-strand F1 of the N-terminal β-barrel in the FimC structure. Interestingly, their side chains are protruding into the interdomain cleft and are surface exposed. Furthermore, analysis of the side chain $^1$H–$^{15}$N chemical shifts of both tryptophans upon binding to FimH revealed significant changes in particular for W84 (Pellecchia et al., 1999).
Residues L103 and A106 are located in the F1-G1 loop that forms an extension of β-strand G1. In the FimC/FimH crystal structure, in the NMR data on the FimC/FimH complex as well as in the complex of PapD and the PapG-peptide, these residues significantly contribute to the intermolecular contacts with the substrate (Kuehn et al., 1993; Choudhury et al., 1999; Pellecchia et al., 1999).

The basic residues R110 and K112, both present in the β-strand G1 of FimC were also randomized simultaneously. Interactions of residues in this strand with hydrophobic solvent accessible side chains of the F strand in FimH make up 60% of the complete binding area in FimC (Choudhury et al., 1999). Moreover, K112 was also found to make a charged hydrogen bond to the carboxy-terminus of the pilus subunits and thus fixes the final β-strand of the substrate in the chaperone cleft.

To analyze the FimC variants that are resulted from five fimC libraries phenotypically, the fimC deficient strain W3110ΔfimC was constructed from the W3110 wild type strain by allelic exchange. W3110ΔfimC was then analyzed by a yeast agglutination assay and electron microscopy. The yeast agglutination is based on the presence of sufficient amounts of the adhesin FimH on the bacterial surface which specifically recognizes mannose structures in the yeast cell wall and leads to the agglutination of type 1 pilus bearing E. coli cells with yeast cells (Krogfelt et al., 1990; Ponniah et al., 1991). Figure 3 and Figure 5A show that bacteria lacking FimC are not longer able to agglutinate with yeast cells due to the complete loss of functional type 1 pili. Complementation of the knock out strain by supplying fimC on a plasmid restores the bacterial wild type phenotype (Figure 4A).

The fimC deficient strain W3110ΔfimC was transformed with the plasmid libraries for the described FimC variants. Between 110 and 450 clones from each of the five random mutagenesis experiments were tested for agglutination with yeast cells (Table 1) and agglutination intensity was classified into four different categories (Figure 3). It is important to note that the decrease in agglutination strength gives no reliable information about the bacterial phenotype with respect to type 1 pilus assembly and subunit stoichiometry and that agglutination only tests for the presence of functional FimH on the bacterial surface. However, since the randomized residues were shown to be directly involved in subunit recognition by TROSY NMR (Pellecchia et al., 1999) and X-ray crystallography (Choudhury et al., 1999), it is most likely that amino acid exchanges in FimC influence the efficiency of chaperone/subunit interaction. The ratio between agglutinating and non-agglutinating clones from each of the mutagenesis experiments, gives a hint about the convertibility of this/these residues without completely lost of FimC stability and/or function (Table 1).
About 120 chosen clones of each mutagenesis were then selected such that the ratio of active and inactive clones was approximately 1:1, and the corresponding *fimC* genes were sequenced. The sequencing data were combined with the different agglutination intensities of the clones and evaluated according to the following scheme: Amino acids were ordered from charged over polar to hydrophobic side chains in two-dimensional plots for each of the four two-residue randomization and the agglutination intensity for each FimC variant was indicated in the plots (Figure 2). This representation allowed a reasonable judgement of important residues in FimC and possible suppressor mutants for non-functional amino acid replacements. To test whether the effects of *fimC* mutations on the *E. coli* agglutination properties were due to an altered subunit binding site of FimC or due to a lower expression level, about 25 FimC variants from each mutagenesis (5 of each agglutination intensity and 5 inactive variants) were selected and analyzed for the periplasmic FimC level by immunoblotting (Figure 3). For more precise description of the corresponding pilus phenotypes, the level of piliation, the maximum pilus length and the homogeneity of the bacteria with respect to piliation were examined by electron microscopy (Figure 4 and Table 2). To investigate the pilus stoichiometry on bacteria harboring special FimC variants, pili were also immunospecifically labeled with gold-coated anti-FimH and anti-FimA antibodies and analyzed by electron microscopy.

**4.2.5.2 Amino acid replacements in the FimC subunit binding site affect type 1 pilus assembly but do not necessarily abolish the overall chaperone function**

The amino acid replacements at the nine selected positions in the type 1 pilus chaperone FimC resulted in numerous chaperone variants, altered in their capacity to mediate pilus formation in *E. coli* and as a consequence, the ability of *E. coli* to agglutinate with yeast cells. Nevertheless, for each randomized residue/pair of residues variants of FimC were identified that i) fold stable in the periplasm (Figure 3) ii) mediate type 1 pilus assembly to an extent similar to that of the wild type (Figure 4A and Table 2) and iii) allow wild type-like agglutination with yeast cells, implying a correct incorporation of the adhesin FimH at the tip of the pilus (Figure 2 and Figure 5A). Therefore, none of these highly conserved residues alone seems to be absolutely essential for chaperone function.

However, for each of the mutagenized residues also variants were detected with a different level of expressed protein, a different number of piliated bacteria, a lowered number of pili per cell as well as an altered pilus length. Moreover, variants belonging to the same random mutagenesis experiment and mediating similar agglutination intensity were found to exhibit
pilus phenotypes that were either homogenous or diverse when comparing single *E. coli* cells of the same clone (Table 2, Figure 4C, 4D). Finally, we also found single FimC variants whose ability to mediate agglutination with yeast did not correlate at all with the presence of type 1 pili (Table 2).

4.2.5.3 There is no overall correspondence between agglutination intensity and FimC expression

In principle, reduction or loss of agglutination strength is expected to result from a decreased incorporation of FimH into the pili. To test whether such changes in the pilus stoichiometry correspond to the expression level of distinct FimC variants and the resulting agglutination intensity, periplasmic extracts of bacteria were analyzed for the level of FimC. With the exception of variants randomized at residue 80 and to some extent at positions 84/86, all probed FimC variants accumulate in the periplasm at levels comparable to those observed for the complemented *fimC* null strain. Most of the FimC variants, however showed the same expression level as wild type FimC (Figure 3) with a clear tendency for decreased agglutination intensity with non-conservative amino acid replacements (Figure 2). Thus, as expected the amino acid substitutions mostly effected the function, i.e. the interaction with the different pilus subunits, and not the stability of FimC. Since residues R8, L103, R110 and K112 can only be replaced conservatively to support a wild type like-agglutination level (Figure 2), a crucial role of these residues for the chaperone function is suggested.

4.2.5.4 Glutamate 80 replacements mainly reduce the FimC stability

In the case of replacements of E80, the reduced expression levels of the FimC variants correlate with the lowered intensity of agglutination indicating protein destabilization by exchanges of E80. All E80 replacements strongly reduce the agglutination extent compared to the wild type protein, and even the conservative substitution E80D was less functional than the wild type (Figure 2B). Surprisingly, small and uncharged residues like alanine or serine still yielded functional FimC. This demonstrates that the negative charge as well as the size of the side chain 80 is critical for the FimC stability. Overall, a glutamate residue at position 80 is clearly the optimal residue to maintain the stability of FimC.

4.2.5.5 The residue R116 is essential for the formation of the interdomain salt bridge

To investigate the role of the salt bridge R116–D192 which is invariant in all members of the periplasmic chaperone family (Hung et al., 1996; Bonci et al., 1997) we introduced the mutations R116D and D192R by site directed mutagenesis. The variant R116D and the
double variant R116D–D192R were so unstable that they could not be detected in periplasmic extracts of W3110ΔfimC by immunoblotting and yielded a fimC null phenotype (Figure 3F, Table 2). However, the cells still showed few and short pili (Figure 4E, Table 2). In contrast, the FimC variant D192R could be detected in the periplasmic extract, albeit at lower concentrations compared to wild type FimC in W3110 (Figure 3F). R116, like E80 is thus critical for the stability of FimC and more important than D192. This may be due to the neighboring residue E80 which also interacts electrostatically with R116 in wild type FimC (Pellecchia et al., 1998) and possibly as well with R192 in the D192R variant. The variant D192R could also partially restore agglutination of W3110ΔfimC with yeast cells, and showed an increased number of pili per cell compared to the variant R116D and the double variant R116D–D192R (Figures 4D, Table 2).

Since the contact area of the chaperone for the structural pilus proteins is almost exclusively formed by residues of the N-terminal FimC domain (Choudhury et al., 1999; Pellecchia et al., 1999) we also tried to test the isolated N-terminal domain for complementation of FimC deficiency in W3110ΔfimC. As shown by Western blot analysis it was not possible to obtain the N-terminal domain (residue 1-121) in detectable amounts, that proves it alone to be extremely unstable (Figure 3F). Nevertheless, bacteria harbouring the plasmid for the periplasmic expression of the N-terminal FimC domain showed few and short pili (maximum length: 1 μm) (Figure 4E, Table 2). Immunospecific labeling with anti-FimH antibodies of these E. coli cells proved that the type 1 pilus adhesin was still located on the tip of these pili (Figure 5C). In comparison, bacteria transformed with plasmids for the periplasmic expression of the isolated C-terminal FimC domain (residues 128-205) showed a complete fimC null phenotype. Conclusively, minute concentrations of the isolated N-terminal chaperone domain are sufficient to maintain a low level of type 1 pilus assembly with the adhesin incorporated at the tip of the pilus.

4.2.5.6 Pilus formation requires FimC concentration above a certain level

Since distinct FimC variants accumulated in lower amounts compared to those in the W3110ΔfimC/pFimC but resulted in a wild type-like agglutination intensity (Figure 3C), we suppose that a certain FimC concentration is required to maintain wild type-like type 1 pilus expression. Moreover, the FimC level in the E. coli wild type strain W3110 is five times lower compared to that of W3110ΔfimC/pFimC (Figure 3F), although its agglutination intensity is even slightly higher (data not shown). Therefore, a certain fraction of pilus subunits might be trapped in the periplasm by complex formation with FimC in the case that
an excess of the chaperone is present. Deviations from an optimal chaperone concentration by either over-expression or protein destabilization thus appear to hamper efficient subunit assembly.

### 4.2.5.7 Agglutination efficiency does not correlate with pilus phenotypes

Electron microscopy was used to characterize the pilus phenotypes of about 200 cells *E. coli* cells for each of the selected FimC variants summarized in Figure 4 and Table 2. Strikingly, agglutination strength as a measure for FimH/yeast interaction did not correspond to the pilus number per cell. Hence, certain FimC variants are capable of restoring agglutination with yeast to a considerable extent but differ significantly from wild type FimC and other variants to in their ability to assist pilus assembly (Table 2, see R7/R8, T7/S8). In addition, strains were analyzed that show an identical piliation pattern as judged by EM, but are either agglutination positive or negative (Table 2, see A80, K80). Furthermore, pilus length and homogeneity of the assembled pili in different cells of the same clone differ strongly in most of the strains with similar agglutination behavior (Table 2). All these observations suggested an altered stoichiometry of pilus subunits in the pili caused by a disturbed specificity of the chaperone for the individual structural subunits. As subunit recognition by FimC is based on a similar mechanism for all subunits (Choudhury et al., 1999), it is likely that an altered FimC surface lowers or even abolishes the affinity to certain subunits. Moreover, it has been shown that the different FimC/subunit complexes are recognized by the outer membrane protein FimD with different affinity (Saulino et al., 1998). Therefore, subunit binding by the chaperone as well as formation of the ternary complex with the outer membrane usher FimD contribute to sorting and order of incorporation of the structural pilus proteins according to their distinct affinity to the chaperone and to the outer membrane pore protein.

### 4.2.5.8 Mannose recognition does not require FimH integration into the pilus

Surprisingly, we found several FimC variants in all random libraries that unambiguously mediated yeast agglutination but showed extremely few or no type 1 pili (Table 2, gray marked). We therefore assumed that FimH might be present on the surface of these cells independent on type 1 pilus attendance. Using FimH-specific immuno-gold labeling, we could show that bacteria with wild type FimC form pili that have the adhesin exclusively incorporated on the top of the pilus (Figure 5B). *E. coli* cells harboring the FimC variant T7/S8 which do not express type 1 pili at all but still agglutinate with yeast (Table 2), are indeed specifically marked with anti-FimH antibodies on the bacterial surface (Figure 5E). To
exclude an unspecific binding of the gold labeled anti-FimH antibodies to the bacteria, the fimC deficient strain W3110ΔfimC was also treated with anti-FimH antibodies, and did not exhibit any labeling (Figure 5A). Since the type 1 pilus adhesin was shown to have the highest affinity to FimC among all type 1 pilus subunits, it was suggested that FimH primarily initiates pilus assembly and therefore is always found on the pilus top (Kuehn et al., 1993; Saulino et al., 1998). We conclude that subunit binding site in the above FimC variants is modified in a such way that the variants can only efficiently interact with FimH, but no longer with FimA and possibly the other minor subunits. Our data are in agreement with the observations by Klemm et al., (1990) who discovered that bacteria deficient in fimA, yielded a mannose sensitive, haemagglutination (MSHA)-positive but non-piliated phenotype. Nevertheless, yeast recognition by non-piliated bacteria seems to be quite inefficient, as the agglutination level was relatively low in the corresponding strains (Table 2), again in accordance with a 25 fold decrease in mannose binding potential of a fimA− strain (Klemm et al., 1990). This indicates that pili are necessary to present the adhesin at a certain distance from the bacterial surface and improving the accessibility of FimH to mannose-containing receptors. On the other hand the data show that FimH molecules directly presented at the bacterial surface are sufficient for the interaction with mannose-containing moieties on receptor cells, albeit with decreased efficiency.

Similar observations were made by FimA-specific immunogold-labeling of the fimC null mutant W3110ΔfimC. Despite the complete lack of type 1 pili, gold-labeled anti-FimA antibodies were found to specifically bind to the bacterial surface (Figure 5F). This indicates that FimA subunits interact with FimD and even were transported through the pore of FimD without the assistance of FimC but do not assemble into filaments. Accordingly we suppose that in the absence of FimC single FimA molecules or small FimA oligomers autonomously can reach FimD, and somehow attain the bacterial surface. Therefore the pores are closed and severe effects on the bacterial growth are prevented as described for an E. coli strain only expressing FimD (Klemm and Christiansen, 1990). In a control experiment, type 1 pili of the wild type E. coli cells were very efficiently labeled anti-FimA antibodies that appear to reproduce the helical arrangement of the FimA subunits in type 1 pili (Figure 5G).

4.2.5.9 Differences in pilus length correlate with homogeneity of assembled pili

We also found that some of the FimC variants lead to clones that showed a strong heterogeneity of the assembled pili. Accordingly, bacteria harboring the same FimC variant express type 1 pili that are different in number and length (Figure 4C, 4D, Table 2).
Interestingly, there is no correlation between homogeneity and the kind of randomly mutated residues, the level of piliation or the intensity of agglutination. All clones with homogenous type 1 pilus formation in the individual cells showed a maximum pilus length not longer than these of the wild type (1–2 μm), whereas all strains with diverse type 1 pilus formation showed longer pili with up to 5 μm (Figure 4A, 4C). The composition of these extraordinary long pili has not yet been investigated but we suppose that the incorporation of one of the minor subunits FimF, FimG or FimH is lowered. This can be explained by the fact that deficiency in fimF and fimG leads to less and longer pili, respectively and the double mutant, however, shows a combined pilus phenotype (Russell and Orndorff, 1992). Moreover, a fimH deficient strain forms less pili that are longer comparable to the wild type strain (Sebbel et al., unpublished results). In the case of W3110ΔfimC harboring FimC M110/K112, antibodies against FimH seem efficiently bind both to pili with wild type length as well as to longer pili (Figure 5D). Therefore, a change in the content of FimF or FimG appears to be the most likely effect of FimC variants in strains with diverse cellular pilus distribution.

4.2.5.10 FimC contact area is formed by residues differently involved in subunit recognition

Our experiments revealed that particularly the residues R8, L103, R110 and K112 of the N-terminal FimC domain contribute to the fine-tuned assembly of type 1 pili in E. coli, since replacement of these amino acids result in a most strongly altered pilus phenotype and adhesiveness. Our detailed investigations on several chaperone variants give an overview on the importance of single chaperone residues in this process.

Regarding the mutations of residues T7 and R8 located in β-strand A1”, it is quite remarkable that strong variations of residue 7 but only conservative substitutions of R8 are tolerated (Figure 2A). Elimination of the positive charge at position 8 had a drastic influence on the E. coli phenotype by reducing the type 1 pilus level due to disturbed hydrogen bonding with the C-terminal carboxylate group of the subunits (Table 2). This is in agreement with the finding that with shortening or extension of the C-terminus of FimH, the ability to bind to FimC is completely lost (Sebbel et al., unpublished results).

Immunoblot analysis showed that amino acid replacements of residue E80 which is located in the interdomain cleft of FimC, mainly lower the overall protein stability, most likely by affecting the formation of the highly conserved salt bridge R116–D192 (Figure 3B).

FimC variants with substitutions of residues W84 and N86 surprisingly had a very small effect on type 1 pilus assembly (Figure 2C). Variants with aromatic residues at position 84 are
expressed in the periplasm to the same extent as wild type FimC, but even a lower FimC level is sufficient for type 1 pilus assembly in the case of these FimC variants (Figure 3C, Table 2). Interestingly, most of the FimC variants with replacements at residues 84 and 86 yielded bacteria with significantly lowered agglutination intensity with yeast. Nearly all of the found inactive variants show proline residues at position 84 or 86 (Figure 2C). This is most likely due to a strong destabilization of FimC (Figure 3C).

An efficient presentation of FimH on the bacterial surface is strictly dependent on the solvent exposed chaperone residue L103. Since only conservative changes to valine or isoleucine are tolerated, hydrophobic residues in this position seem to be essential for a strong interaction with residues of the C-terminal β-strand of the subunit (Choudhury et al., 1999). In contrast, A106 is interchangeable with even negatively charged residues like glutamate, showing only a slight reduction in pilus assembly (Figure 2D).

R110 and K112, belonging to β-strand G1, are highly conserved amino acids in functional FimC variants (Figure 2E). It is remarkable that there is a severe dependency on positive charges at residues 110 and 112, although in both positions changes to almost all amino acids are tolerated if one of the two residues is either an arginine or a lysine. The finding that variants harboring a positive charge at position 110 but small and uncharged amino acids (G, A) at position 112 are still able to fulfill the chaperone function, gives a hint that interaction with the C-terminal carboxylate of the subunits does not exclusively depend on K112. We believe that efficient binding to free carboxylate at the C-terminus of subunits is mainly realized by R8.

In summary, this study shows that numerous exposed residues in the subunit binding site of the chaperone FimC contribute to the specificity of type 1 pilus subunit recognition, the key step in the process of type 1 pilus assembly in E. coli. It was demonstrated that each of the nine mutagenized amino acids (T7, R8, E80, W84, N86, L103, A106, R110, K112) may be replaced by other residues without complete loss of functional type 1 pilus formation. We could demonstrate that effective interaction with the subunits is strictly dependent on residues R8 and L103, and indirect on E80 that guarantees protein stability. Since certain FimC variants caused a non-piliated, but yeast agglutination positive E. coli phenotype, and other FimC variants led to wild type-like pili as well as pili with a 2 fold length, we conclude that a modified contact area in the chaperone alters fine-tuned specificities of the chaperone for the individual pilus subunits that determine pilus stoichiometry, assembly initiation and pilus assembly itself. This is the first demonstration that a molecular chaperone determines the stoichiometry and morphology of a macromolecular hetero-oligomeric protein structure.
Figure 1. Ribbon drawing of the NMR structure of FimC. The side chains of residues that were randomized simultaneously are depicted with the same color. The figure was generated with the program MOLMOL (Koradi et al., 1996).
Figure 2. Identification of residues in the binding site of FimC that are important for type 1 pilus biogenesis. Nine residues of FimC were subjected to random mutagenesis, either pairwise (A) T7/R8; (C) W84/N86; (D) L103/A106 and (E) R110/K112 or alone (B) E80. The 5 corresponding expression plasmid libraries encoding the FimC variants were used to transform the fimC null strain W3110ΔfimC. Clones of transformed cells were tested for the presence of type 1 pili by their ability to agglutinate with yeast cells. Overall, 333 “active” and 217 “inactive” FimC variants (lacking stop codons or frame shifts) were sequenced (Table 1). The 20x20 matrices correspond to the 400 combinations of the pairwise randomizations. The amino acids in the matrices are ordered as follows: Basic, acidic, polar and hydrophobic residues (with increasing size). The wild type sequence (WT) of each randomized residue is indicated in bold face. The intensity of agglutination with yeast cells was classified into four different categories. Black squares represent a strong agglutination like that observed for W3110ΔfimC complemented with wild type FimC, and squares in dark gray, gray and light gray represent decreasing agglutination levels. (X) indicates that no agglutination was observed. Bacterial growth and the agglutination assay were performed according to Experimental procedures.
**Random Mutagenesis of FimC**

### A

<table>
<thead>
<tr>
<th>Residue 7 / Residue 8</th>
<th>Agglutination with yeast</th>
<th>FimC knock out</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/R</td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td>L/R</td>
<td>++++</td>
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</tr>
<tr>
<td>T/K</td>
<td>++++</td>
<td>FimC knock out</td>
</tr>
<tr>
<td>V/R</td>
<td>++++</td>
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</tr>
<tr>
<td>S/R</td>
<td>++++</td>
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<tr>
<td>M/R</td>
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<tr>
<td>S/K</td>
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<td>R/L</td>
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### B

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### C

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<tr>
<td>T</td>
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<tr>
<td>S</td>
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### D

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<th>FimC knock out</th>
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<td>I/S</td>
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<td>V/S</td>
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<td>I/T</td>
<td>++++</td>
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</tr>
<tr>
<td>L/I</td>
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</tr>
<tr>
<td>L/E</td>
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</tr>
<tr>
<td>M/T</td>
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</tr>
<tr>
<td>L/P</td>
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</tr>
</tbody>
</table>

**Legend:**
- **FimC:** protein of interest
- **Agglutination with yeast:** indicates the interaction strength with yeast, with ++++ indicating strong interaction.
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</tr>
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<td>R/C</td>
<td>L/S</td>
<td>E/G</td>
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<td>++++</td>
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<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
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<td>L/A</td>
<td>Q/T</td>
<td>K/Y</td>
<td>R/W</td>
<td>S/T</td>
<td>D/H</td>
<td>M/A</td>
<td>Y/S</td>
<td>K/L</td>
<td>T/H</td>
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<tr>
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<td>++++</td>
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</tbody>
</table>

Figure 3. Immunoblot analysis of periplasmic expression levels of FimC variants obtained after random mutagenesis. For each of the 5 different random mutagenesis experiments ((A) T7/R8; (B) E80; (C) W84/N86; (D) L103/A106 (E) R110/K112) about 5 different variants from each class of agglutination intensity were selected arbitrarily and tested for the periplasmic FimC level. Cells of *E. coli* W3110ΔfimC expressing the FimC variants were grown as described in Experimental procedures, periplasmic fractions were separated on a 15% polyacrylamide-SDS gel, blotted onto a PVDF membrane, and FimC was immunospecifically stained with polyclonal rabbit anti-FimC antibodies. The amino acids at the randomized positions are indicated above the bands, as well as the classification of the corresponding FimC variant in the yeast agglutination assay. The four categories of agglutination are the same as those in Figure 2, but are represented by (+++), (+++), (++), and (+) instead of the black and grey squares in Figure 2. (−) corresponds to no agglutination ((X) in Figure 2). As a control, a sample of the periplasmic extract of W3110ΔfimC and of W3110ΔfimC expressing wild type FimC was applied to each gel (right and left lanes, respectively), wild type residues are given in bold face. (F) Immunoblot analysis of different FimC variants from (1) *E. coli* W3110; (2) W3110ΔfimC; (3–9) W3110ΔfimC transformed with plasmids for periplasmic expression of (3) FimC (pFimC); (4) the N-terminal FimC domain (pFimC-N) (residue 1–121, 14 kDa); (5) the C-terminal FimC domain (pFimC-C) (residue 128–205, 8.8 kDa); (6) FimC–R116D (pFimC–R116D); (7) FimC–D192R (pFimC–D192R); (8) FimC–R116D/D192R (pFimC–R116D/D192R).
Figure 4. Different FimC variants result in novel type 1 pilus phenotypes. Electron micrographs of *E. coli* cells were rotary shadowed with 1.5 nm platinum-carbon (95/5). Images represent examples for cells of different pilus expression level: (A) W3110ΔfimCpFimC, a bulk of pili per cell; (B) W3110ΔfimCpFimC-M7/R8, many pili per cell; (C) and (D) W3110ΔfimCpFimC-M110/K112, divers, many and few pili per cell, respectively, W3110ΔfimCpFimC-D192R belongs to the phenotype of (D); (E) W3110ΔfimCpFimC-N, single pili per cell. W3110ΔfimCpFimC-R116D and W3110ΔfimCpFimC-R116D/D192R belong to this phenotype. The scale in (A), (B), (D) and (E) is identical. The vertical bars in (A) and (C) correspond to 1.0 μm.
Figure 5. Different amino acid exchanges in FimC differently affect the interaction with the type 1 pilus subunits FimA and FimH. E. coli cells harboring different FimC variants were immunospecifically labeled with gold-coated polyclonal anti-FimH (A)-(E) or anti-FimA (F) and (G) rabbit antibodies as described in Experimental procedures. (A) W3110ΔfimC; (B) W3110ΔfimC/pFimC; (C) W3110ΔfimC/pFimC-M110/K112; (D) W3110ΔfimC/pFimC-N; (E) W3110ΔfimC/pFimC-T7/S8; bacteria harboring the FimC variants that are marked gray in table 2 belong to this type of anti-FimH antibody labelling; (F) W3110ΔfimC; (G) W3110ΔfimC/pFimC. The vertical bar in (A)–(G) corresponds to 100 nm.
Table 1. Clones tested for yeast agglutination and number of random fimC mutants sequenced.

<table>
<thead>
<tr>
<th>Randomized residues</th>
<th>Clones per μg ss DNA</th>
<th>Clones tested for agglutination with yeast cells</th>
<th>Selected and sequenced fimC genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>active [%]</td>
<td>inactive [%]</td>
<td>total</td>
</tr>
<tr>
<td>T7/R8</td>
<td>6.1 (\times) 10^7</td>
<td>17.6</td>
<td>82.4</td>
</tr>
<tr>
<td>E 80</td>
<td>3.2 (\times) 10^7</td>
<td>34.3</td>
<td>65.7</td>
</tr>
<tr>
<td>W84/N86</td>
<td>5.3 (\times) 10^7</td>
<td>80.3</td>
<td>19.7</td>
</tr>
<tr>
<td>L103/A106</td>
<td>1.8 (\times) 10^7</td>
<td>17.7</td>
<td>82.3</td>
</tr>
<tr>
<td>R110/K112</td>
<td>6.1 (\times) 10^6</td>
<td>29.1</td>
<td>70.9</td>
</tr>
</tbody>
</table>

\(^a\) activity as defined by the ability to agglutinate with yeast cells.

\(^b\) sequenced inactive genes that are corrected for genes with stop codons or frame shifts.
Table 2. Phenotypes of type 1 pilus expression and yeast agglutination intensity of *E.coli* strain W3110Δ*fimC* harboring distinct FimC variants.

<table>
<thead>
<tr>
<th>Expressed FimC variants</th>
<th>Agglutination with yeast</th>
<th>Fraction of bacteriab</th>
<th>Number of pili per piliated bacterium</th>
<th>Maximal pilus length [μm]</th>
<th>Homogeneous/piliation</th>
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</thead>
<tbody>
<tr>
<td>W3110Δ<em>fimC</em></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>D</td>
</tr>
<tr>
<td>pFimC</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>2</td>
<td>H</td>
</tr>
</tbody>
</table>

**Mutagenesis T7/R8**

| L7/R8                  | +++                      | +++                   | ++                                    | 2                         | H                    |
| T7/K8                  | +++                      | +++                   | ++                                    | 2                         | H                    |
| V7/R8                  | +++                      | ++                    | +++                                   | 2                         | H                    |
| S7/R8                  | +++                      | ++                    | +++                                   | 2                         | H                    |
| M7/R8                  | +++                      | +++                   | +++                                   | 2                         | H                    |

| S7/K8                  | +                        | +++                   | +                                     | 1                         | H                    |
| C7/R8                  | +                        | +++                   | +                                     | 2                         | H                    |
| A7/K8                  | +                        | +++                   | +                                     | 3                         | D                    |
| A7/R8                  | +                        | +++                   | +                                     | 3                         | D                    |

| R7/R8                  | +                        | +++                   | ++                                    | 1                         | H                    |
| T7/S8                  | +                        | -                     | -                                     | -                         | -                    |
| R7/L8                  | +                        | (+)                   | +                                     | 1                         | -                    |
| H7/R8                  | +                        | (+)                   | +                                     | 1                         | -                    |
| L7/K8                  | +                        | (+)                   | +                                     | 3                         | -                    |

| T7/Q8                  | +                        | (+)                   | +                                     | 2                         | -                    |
| P7/K8                  | +                        | -                     | -                                     | -                         | -                    |
| V7/I8                  | +                        | (+)                   | +                                     | 2                         | -                    |
| T7/M8                  | +                        | (+)                   | +                                     | 1                         | -                    |
| T7/A8                  | +                        | (+)                   | +                                     | 1                         | -                    |

| L7/S8                  | -                        | -                     | -                                     | -                         | -                    |
| T7/L8                  | -                        | -                     | -                                     | -                         | -                    |
| H7/K8                  | -                        | (+)                   | +                                     | 2                         | -                    |
| R7/K8                  | -                        | -                     | -                                     | -                         | -                    |
| S7/T8                  | -                        | -                     | -                                     | -                         | -                    |

**Mutagenesis E80**

| A80                    | +++                      | (+)                   | +                                     | 1                         | -                    |
| T80                    | +++                      | (+)                   | +                                     | 1                         | -                    |
| D80                    | +++                      | +++                   | +++                                   | 4                         | D                    |

| C80                    | +                        | ++                    | ++                                    | 1                         | H                    |
| S80                    | +                        | (+)                   | +                                     | 5                         | -                    |

| Q80                    | +                        | -                     | -                                     | -                         | -                    |
| I80                    | -                        | -                     | -                                     | -                         | -                    |
| F80                    | -                        | -                     | -                                     | -                         | -                    |
| R80                    | -                        | -                     | -                                     | -                         | -                    |
| K80                    | -                        | (+)                   | +                                     | 2                         | -                    |
| Y80                    | -                        | (+)                   | +                                     | 2                         | -                    |
| H80                    | -                        | -                     | -                                     | -                         | -                    |
| M80                    | -                        | (+)                   | +                                     | 2                         | -                    |
| N80                    | -                        | (+)                   | +                                     | 2                         | -                    |
| G80                    | -                        | -                     | -                                     | -                         | -                    |
### Random Mutagenesis of FIMC

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<td>(+)</td>
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Bacteria were examined for the number of pili, maximal pilus length and the fraction of cells bearing pili in static cultures. At least 400 cells for each expressed FimC variant were analysed by electron microscopy.

a as classified in Figure 2

Piliated bacteria are enriched against non-piliated in static cultures. (+++) corresponds to the fraction of piliated cells corresponding to that observed for the wild type FimC. (++), many; (++), few; (+), single; (-), no cell bear type 1 pili.

b as defined in Figure 4
4.2.6 References


During this PhD-thesis the biochemical properties of FimC, the periplasmic pilus chaperone of type 1 pili from *Escherichia coli* were investigated by *in vitro* experiments and the function of FimC in the process of pilus formation was analyzed in detail. Based on structural studies, individual regions and distinct residues of the protein were investigated for their role in the interaction with structural pilus subunits. In addition, the *in vivo* analysis of a multitude of different FimC variants shed new light on the molecular events during assembly of these highly organized, heteropolymeric protein structures in *E. coli*. Specifically, the examination of bacterial and pilus phenotypes caused by chaperone sequence variations broadened the understanding of the energy-independent assembly of type 1 pili.

Biosynthesis of type 1 pili in *E. coli* is one example for effective co-operation of a set of specialized assembly factors. One of these determinants is the periplasmic two-domain protein FimC that assists the well-ordered assembly of type 1 pili at the cell surface. In the first chapter of part four in this thesis, the first biochemical characterization of a bacterial pilus chaperone was described, using FimC as a model protein. For this purpose, FimC was overproduced in the periplasm of *E. coli* and characterized with respect to spectroscopic properties and stability against denaturants. Furthermore, the contribution of each of the two domains to the overall protein stability was investigated. While the N-terminal domain proved to be completely unstable *in vivo*, and could not be obtained in detectable amounts, the C-terminal domain could be overproduced but formed cytoplasmic inclusion bodies. Comparison of the thermodynamic stability of the C-terminal domain with that of the full-length protein revealed a 10-fold decreased energy of folding. As no domain-wise unfolding of FimC was observed, both domains in FimC appear to have evolved towards mutual stabilization so that the intact chaperone shows a two-state behavior of folding.

The main interest in pilus biosynthesis was focused on the molecular mechanism of the chaperone's interaction with the structural pilus subunits to understand how a single factor coordinates the assembly of different proteins into a highly ordered macromolecular structure. The starting point for the present investigation was the model proposed by Kuehn et al., (1993) according to which binding of the chaperone
to the individual subunits is strictly dependent on the strongly conserved C-terminal segment of the unfolded pilus subunits. To investigate the importance of the C-termini of the pilus proteins for chaperone binding, a synthetic peptide corresponding to the 11 C-terminal residues of the adhesin FimH was synthesized, and affinity towards FimC was measured. The relatively high dissociation constant (6 μM) demonstrates a very weak binding which makes chaperone recognition only via the C-terminal amino acid stretch quite unlikely. In addition, randomization of conserved residues in the C-terminal part of FimH illustrated that exchange of the conserved hydrophobic residues in the C-terminal motif did not abolish type I pilus formation and consequently do not have a detectable influence on the interaction with the chaperone (Sebbel et al., unpublished results). However, either elongation or shortening of the subunit at its C-terminus led to complete loss of piliation. In addition, as shown by in vitro experiments on the aggregation of FimH, FimC exclusively interacts with folded FimH (Sebbel et al., unpublished results). This together with the low affinity of FimC for the C-terminal peptide of FimH, almost certainly excludes a contribution of FimC to subunit folding.

In the second part of this thesis, the role of single residues or parts of FimC for subunit binding was investigated by in vivo studies. For this purpose, an E. coli fimC null mutant was constructed that proved to be completely incompetent of type 1 pilus assembly. This defect could be restored by transformation of the null mutant with an expression plasmid for FimC, which was the prerequisite for the following studies on randomly mutated FimC variants. On the basis of our NMR measurements on the residues in FimC that interact with FimC (Pellecchia et al., 1999) and the subsequent X-ray structure determination of the FimC/FimH complex (Choudhury et al., 1999), it became evident that many conserved and invariant chaperone residues from its N-terminal domain define the subunit binding site of the chaperone. By random mutagenesis of nine of these residues, FimC was investigated for its "tolerance" of amino acid changes. We discovered that the invariant residues R8 and K112, which interact with the C-terminal carboxylate groups of the subunits, were the most important residues for the function of FimC, while other amino acid exchanges had a strong influence on pilus assembly and stoichiometry. This confirms that the chaperone-subunit interaction requires a large binding surface involving many residues the the chaperon, in accordance with the view that FimC recognizes the
tertiary structure of the subunits. Since most of the FimC mutations had no influence on expression level of FimC the analyzed amino acid replacements mainly influenced the FimC/subunit interaction so that clear-cut information on the functional role of the mutated residues could be obtained. As a large number of FimC variations still yielded functional pili, it appear that a relatively weak chaperone/subunit complex stability is sufficient for pilus formation. Complex formation in the periplasm on the one hand prevents unproductive polymerization prior to translocation through the outer membrane and on the other allows a targeted delivery of the subunits to the correct assembly platform.

During our mutagenesis study, FimC variants were selected that present FimH independent on functional type 1 pilus assembly on the cell surface. It has been suggested previously that pilus chaperones direct the subunit incorporation rate by showing fine-tuned affinities for the different pilus proteins (Kuehn et al., 1993). Consequently, the FimC variants that disrupt pilus assembly but still allow transport of FimH through the pore of FimD, appear to have lost the ability to bind to FimA, but still recognize FimH. In general, it is likely that most of the FimC variants generated in this study possess different relative affinities for the individual type 1 pilus subunits compared to wild type FimC. FimH is supposed to bind to FimC with the highest affinity of all type 1 pilus subunits, and the FimC/FimH complex was shown to interact with FimD more efficiently than the other complexes (Saulino et al., 1998). Thus, FimH is favored for translocation through the outer membrane which may explain that it is exclusively found at the pilus tip. A high affinity for FimH would also explain that mutations in FimC are the best tolerated in the case of the FimC/FimH complex. In bacteria that present FimH in the absence of the pilus rod, the accessibility to host cell receptors must stereo-specifically hampered which agrees with the fact that they quite inefficiently agglutinate with yeast cells.

Although the tested FimC residues show a wide diversity in the kind of amino acids that are still sufficient for the interaction with subunits, certain residues are restricted to conservative replacements. Beside the above mentioned residues R8 and K112, the surface exposed hydrophobic side chain L103 appears to be particularly important for type 1 pilus assembly and most likely for the interaction of FimC with all subunits. Our results together with the finding by Sebbel et al., (unpublished results) that elongation or shortening of the subunits at the C-terminus completely prevents formation of the complex with FimC and therefore type 1 pili formation,
clearly show again that interaction of the subunit C-terminal carboxylate with charged residues in FimC is absolutely essential in this process.

In another set of experiments, the role of the highly conserved inter-domain salt bridge for functional stability of FimC was examined. The salt bridge is formed by R116 and D192 and was found to direct the relative domain orientation (Hung et al., 1996; Pellecchia et al., 1998). In vivo studies of the FimC variants R116D, D192R and R116D/D192R revealed the important role of R116 for FimC stability, since only the variant D192R was expressed in detectable amounts in the periplasm, albeit in a five-fold lower concentration compared to the wild type protein. R116 seems thus to be much more important for protein stability than D192, which might be explained by complementation of negative charge deficiency by the neighboring residue E80 (Pellecchia et al., 1998). Random mutagenesis of residue 80 also demonstrated that a negative charge and a small side chain are critical for the stability of FimC. Our results are comparable to pulse chase experiments on the expression of corresponding salt bridge variants of the related P pilus chaperone PapD that also were found to be destabilizing and caused subsequent protein degradation in the periplasm (Hung et al., 1999). A role of the salt bridge in subunit recognition can also not be excluded, as binding of certain subunits to the chaperone may change the relative orientation of the domains and thus the geometry of the salt bridge.

Since X-ray structure and NMR measurements revealed entirely the N-terminal chaperone domain responsible for substrate binding, the N-terminal domain was tested for its ability to fulfill the chaperone function in vivo. Whereas in the yeast agglutination assay cells show a fimC null phenotype, electron microscopy demonstrated very few and shortened pili with a length of only up to 1 μm. Thus, pilus assembly had been started but was very limited, most likely because the N-terminal domain is readily degraded by periplasmic proteases. This again illustrates the importance of intradomain interactions for the entire stability of FimC.

In the next following sentences all data on type 1 pilus formation and the function of FimC including the findings of this thesis are summarized.

Pilus subunits newly transported to the periplasm are oxidized by DsbA, and after disulfide bridge formation, they fold autonomously to an assembly-competent tertiary structure. Since the folded subunits display interactive surfaces, spontaneous oligomerization in the periplasm is started. The formation of subunit oligomers is
controlled on the one hand by periplasmic pilus chaperones and on the other by periplasmic proteases. The pilus chaperone specifically interacts with single subunits, thereby preventing further polymerization by capping the subunit's interactive surfaces. However, as found in this study complex formation seems to be controlled by different affinities of the chaperone for the various subunits, even though the binding mechanism is essentially the same. Additionally, individual chaperone-subunit complexes also differ in their affinity to the outer membrane usher FimD. Therefore, the concentration of distinct chaperone-subunit complexes as well as their affinity to the polymerization platform appears to define the final pilus composition. Since the chaperone alone does not bind to FimD, the recognition might be due to surface residues of the bound subunit alone or a simultaneous recognition of epitopes of both FimC and the bound subunit.

After complex dissociation and subunit translocation across the outer membrane, possibly as linear, fimbrillum-like fibers, additional subunit-subunit interactions outside the cell lead to the formation of the helical pilus rod that simultaneously may drive transport of further pilus subunits from the inner side of the outer membrane. How the pilus length is defined and whether there is a mechanism to remove pili from the surface, is presently not known.

In the last part of the discussion, I want to give an outlook on experimental approaches that may shed light on events during pilus biogenesis which are not completely understood.

The present study strongly suggests that the interaction between the chaperone FimC and all type 1 pilus subunits is based on the same binding mechanism but with different affinities. In vitro experiments that allow the measurement of the dissociation constants between FimC and the single subunits would be very helpful to prove this assumption. Moreover, attempts of in vitro reconstitution of type 1 pili by reassembly of purified pilus subunits in the presence and absence of FimC would certainly give hints about binding preferences of the single pilus proteins and the role of FimC in the subunit/subunit recognition during pilus formation.

Electron microscopy analysis of type 1 pili from strains deficient in one of the minor subunits by immunolabeling with antibodies against all the different minor subunits would answer the question of whether the absence of one of subunits hampers the incorporation of another. These experiments may also clarify the exact order of subunit arrangement in the pilus. Certain concentrations of one of the minor
subunits for example could be required to maintain the initiation of pilus assembly or the natural pilus length, while deviations or failure should result in altered pilus phenotypes.

Since we have found that a FimC concentration below the wild type level is sufficient for a maximum type 1 pilus expression, the influence of a lower chaperone concentration on the bacterial phenotype could also be investigated by titrating FimC via an inducible promoter in a fimC deficient strain. It would be interesting to see whether a minute chaperone concentration results in a change of pilus number and length. If the main function of the protein is to keep the level of monomeric subunits in the periplasm low (Sebbel, 1999), small concentrations of FimA in the periplasm might give the same phenotype.

Although the formation of type 1 pili is completely abolished in the absence of the chaperone, FimA was still present at the surface. This indicates that single FimA molecules or small FimA oligomers can interact with FimD without chaperone assistance. Investigations of a ΔfimA/ΔfimC strain by immunolabeling would clarify whether other subunits are able to interact with FimD, in a chaperone-independent manner avoiding a severe bacterial phenotype.

From a therapeutical point of view towards the development of drugs against uropathogenic E. coli cells several different strategies can principally be followed. On the one hand the process of functional type 1 pilus assembly could be abolished by developing drugs that are able to block the subunit binding site of FimC by irreversible complex formation. Another possibility would be to block the lectin binding site in FimH on the tip of type 1 pili during an infection. A very promising approach in this context appears to the development of single chain antibody Fv fragments that cover the mannose binding site of the lectin domain of FimH. A particular advantage of scFvs would be that they can freely pass the blood-urine barrier due to their low molecular mass and thus would principally be able to competitively elute infectious E. coli cells from infected epithelium cells. Such scFvs with high affinities and specificities for FimH can be selected by the phage display method.


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Pilus chaperone
FimC–adhesin FimH
interactions mapped by
TROSY-NMR

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The 23 kDa two-domain periplasmic chaperone FimC from Escherichia coli is required for the assembly of type-1 pilus, which are filamentous, highly oligomeric protein complexes anchored to the outer bacterial membrane that mediate adhesion of pathogenic E. coli strains to host cell surfaces. The establishment of many bacterial infections is related to the presence of adhesive cell organelles on the bacterial surface, such as pili or fimbriae. The biogenesis of pili requires the presence of specialized periplasmic assembly factors (chaperones) that bind individual pili subunits in the periplasm and deliver them to an assembly platform in the outer membrane. The biogenesis of pili involves specific periplasmic assembly factors (chaperones) that bind individual pili subunits in the periplasm and deliver them to an assembly platform in the outer membrane. The pilus chaperone FimC3 provides the basis for studies of the interaction between FimC and the 28 kDa mannose-binding type-1 pilus subunit FimH. The FimC–FimH complex was then shown to be long-lived on the NMR chemical shift time scale (here, 1 > 2 ms) chosen in the absence of resonance assignments for bound FimC6. In this study we applied transverse relaxation-optimized spectroscopy (TROSY)1 to obtain a [15N,'H]-TROSY spectrum of FimC, which is responsible for the binding of the 28 kDa mannose-binding type-1 pilus subunit FimH by 'H NMR chemical shift mapping, using transverse relaxation-optimized spectroscopy (TROSY). The FimC–FimH complex is formed nearly entirely by the N-terminal domain, and its extent and shape indicate that FimC binds a folded form of the pilus subunits. Chemical shift mapping with TROSY
The recently determined NMR structure of the 205-residue protein FimC provided the basis for studies of the interaction between FimC and the 28 kDa mannose-binding type-1 pilus subunit FimH. The FimC–FimH complex was then shown to be long-lived on the NMR chemical shift time scale (here, 1 > 2 ms)1 by analyzing a solution of 0.3 mM unlabeled FimH and 0.4 mM [15N]-labeled FimC. In this sample two separate sets of resonances were observed in [15N–'H]-TROSY correlation spectra, one corresponding to free FimC and the other to FimC in the complex with FimH. Using transverse relaxation-optimized spectroscopy ([15N,'H]-TROSY), complete [15N–'H] fingerprint1 of FimC with 205 [15N–'H] correlations (192 backbone [15N–'H], 2 Trp indole [15N–'H] and 11 Arg [15N–'H]; Fig. 2a), were then recorded for both free [15N,1'5]-labeled FimC (Fig. 1a) and the 51 kDa FimC–FimH complex containing uniformly [15N,1'5]-labeled FimC bound to unlabeled FimH (Fig. 1b). The new potentialities of TROSY1 to record high-resolution NMR spectra of very big particles in solution1 thus enabled a chemical shift mapping of FimC contacts in the supramolecular structure of the FimC–FimH complex. Conventional NMR correlation spectroscopy with the [15N,1'5]-FimC–FimH complex had resulted in observation of only ~140 of the expected 205 [15N–'H] correlations, mostly as broad lines (data not shown), which would make a structural analysis highly speculative. In contrast, the complete data set obtained with TROSY enabled the construction of a FimC surface map of lower limits on the interaction-induced chemical shifts, based on sequence-specific NMR assignments for free FimC. In this way the first mapping of intermolecular contacts for a periplasmic chaperone complex with an intact pilus subunit was obtained, since even crystallographic data have so far been limited to studies of a PapD complex with a synthetic model peptid7.

FimC–FimH recognition
A surface map of lower limits on the chemical shift differences between free and bound FimC was generated as follows: Superposition of the [15N,'H]-TROSY spectra of FimC and the FimC–FimH complex (Fig. 1a) showed that there is a large number of peaks with nearly identical shifts and similar relative peak amplitudes in the two spectra, indicating 1:1 correspondence with free FimC for the assignment of these resonances of bound FimC. On this basis we concluded that the weighted average of the backbone [15N–'H] chemical shift variations upon binding to FimH, 3Dw, provides a good index for the recognition motif for bacterial pilus chaperones7. For another subunit, the 28 kDa mannose-binding type-1 pilus subunit FimH, of this complex the PapD structure is very similar to the NMR structure of FimC (Fig. le,f) shows that all residues with 3Dw > 0.1 p.p.m. were actually in contact with FimH. However, a mapping of the results of this tentative procedure on the three-dimensional structure of FimC (Fig. le,f) shows that all residues with 3Dw > 0.1 p.p.m. are located on the surface of FimC. This is compatible with preservation of the conformation of FimC upon binding to FimH and the assumption that the observed chemical shift differences are a consequence of direct interactions with FimH. On the basis of the steadiness of the folds of the individual FimC domains1, one might have predicted that they would be preserved in the complex. The absence of chemical shift changes for the [15N–'H] resonance of Arg 116, which largely defines the relative orientation in unliganded FimC by a salt bridge with Glu 80 and Asp 102, further supports that the relative arrangement of the two domains is also preserved. The apparent internal consistency of the results indicates that the approach chosen in the absence of resonance assignments for bound FimC is appropriate for the FimC–FimH complex.

FimC binds folded subunits
The strongly conserved C-terminal segment of the subunits from different adhesive pili has been suggested to be a common recognition motif for bacterial pilus chaperones7. For another member of this chaperone family, PapD, the X-ray structure is available for a complex with a synthetic peptide corresponding to the C-terminal 19 residues of the P-pilus subunit PapC. In this complex the PapD structure is very similar to the NMR
Fig. 1 Comparison of $^{15}$N-H-TROSY spectra of uniformly $^{15}$N-H-labeled FimC in a, the free form and in b, the 1:1 complex with the unlabeled mannose-binding type-1 pilus subunit FimH. The two spectra were recorded with identical conditions (0.4 mM $^{15}$N-H-labeled FimC in 90% H$_2$O/10% D$_2$O, pH 5.0, 30 °C). a. Superposition of a region of the spectra in (a) and (b) (blue free FimC; red, FimC complexed with FimH). b. Superposition of the spectral region containing the $^{15}$N-H resonances of the Arg side chains. These two spectra resulted from different $^{15}$N-H-TROSY experiments, where the $^{15}$N carrier position was set to 85 ppm in order to optimize $^{15}$N observation. In (c) and (d), the blue crosspeaks are labeled with the sequence-specific assignments for free FimC, using one-letter amino acid symbols and the sequence positions. The connections between red and blue peaks indicated by black lines are discussed in the text. e. Projection of the chemical shift mapping of the FimC-FimH contacts onto the three-dimensional structure of FimC. A stereo view of the polypeptide backbone in the NMR structure of FimC is afforded by a spline function drawn through the C$\alpha$ positions. Chemical shift variations due to FimH binding, $\Delta\delta c$, are indicated by the following color code: gray, $\Delta\delta c > 0.3$ p.p.m.; yellow, 0.1 p.p.m. $< \Delta\delta c < 0.2$ p.p.m.; orange, 0.2 p.p.m. $< \Delta\delta c < 0.3$ p.p.m.; red, $\Delta\delta c > 0.3$ p.p.m. $\Delta\delta c$ is a weighted average of the $^{15}$N and $^{1}H$ chemical shifts: $\Delta\delta c = 0.5(\Delta\delta^{15}N) + 0.5(\Delta\delta^{1}H)$. f. Same as (e) after rotation by 90° about a horizontal axis so that the two 'wings' of the structure point toward the viewer. The molecular models were drawn with MOLMOL [22].

Mapping onto the three-dimensional FimC structure of all residues with $\Delta\delta c > 0.1$ p.p.m. shows that only residues of the N-terminal domain and in the cleft between the N- and C-terminal domain interact with FimH (Figs 1e, f, 2a, c), whereas the C-terminal domain is essentially not involved in pilus subunit binding. Thus, in contrast to a previous hypothesis, the C-terminal domain is unlikely to promote pilus subunit folding. Analysis of side-chain $\Delta\delta c$ values (Fig. 1d) revealed significant shifts for the solvent-exposed residues Trp 36 and Trp 84, and for Arg 8 and Arg 110, which form a basic patch near Lys 112 in the structure of FimC [22, d]. It is most intriguing, however, that the contact area between FimC and FimH is much larger than that defined by intermolecular contacts in the aforementioned PapD-peptide complex, and that it involves numerous residues outside of the surface area corresponding to the peptide binding site of PapD (Fig. 2c, d). Consequently, FimC must recognize additional structural epitopes to those anticipated from the PapD-peptide crystal structure, indicating a folded state of FimH in the complex with FimC. A folded state of FimH is further indicated by the complete formation of both disulfide bonds in FimH, high stability of complexed FimI against degradation in vitro, high solubility of the FimC-FimH complex, and high stability of the complex against aggregation in vitro and in vivo.

**FimC-assisted pilus assembly**

Knowledge of the binding surface of FimC for its pilus subunit FimH sheds new light on the molecular basis of chaperone-mediated assembly of bacterial pili. First, it shows that the chaperone does not only interact with the highly conserved C-terminal segment of the pilus subunit but specifically recognizes much wider surface regions. As the different subunits of a given...
pili show significant sequence homology also outside of their C-terminal segments. FimC may interact with the other four subunits of type 1 pili—that is, FimA, FimE, FimG and FimH—in a manner very similar to that with FimH. Thus, in contrast to most other molecular chaperones that recognize unfolded or partially folded polypeptides with low sequence specificity, FimC appears to bind the folded, assembly-competent pilus subunits to prevent spontaneous association of the subunits in the periplasm, and to mediate the delivery of the individual subunits to the assembly platform at the outer bacterial membrane.

Second, the C-terminal domain of FimC essentially does not interact with the pilus subunit (Figs 1d,e, 2a,b,c). This suggests that the two domains of periplasmic pilus chaperones have different functional roles. The C-terminal FimC domain would thus presumably be required for targeting of the assembly platform at the outer membrane, while the N-terminal domain is responsible for subunit binding. In this way, the unliganded C-terminal domain in the FimC-FimH complex provides a free binding surface for specific interactions with other compounds, such as the assembly platform, which is assumed to trigger the release of the subunit to the assembling pilus. This view is further supported by the experimental observation that periplasmic pilus chaperones specifically interact with their own assembly platforms, and by the high sequence variability of the C-terminal domains when compared with the N-terminal domains (Fig. 2a,b). Type 1 pili are not only involved in the virulence of bacteria but also constitute critical virulence factors and mediate the escape of the bacteria from the immune response of the host and their survival inside of macrophages. Therefore, drugs preventing assembly of functional type 1 pili constitute promising therapeutics for common E. coli infections of the urinary tract, and the identified binding site of FimC for FimH may be useful in the rational development of such compounds.

Methods

Preparation of 15N- and 1H,15N-labeled FimC. E. coli was grown on Martek 9dN medium (97% 1H and >98% 15N, Martek Biosciences Corporation). Adaptation of the bacteria to the medium was achieved by serial cultures grown in 30%, 70% and 97% v/v Martek-9dN in LB medium (10 g trypton, 5 g yeast extract, 5 g NaCl, pH 7.4). All media con-
tained ampicillin (100 µg ml⁻¹). After adaptation a 4:1 culture of 97% Martek 9nD was inoculated 1:10 with an overnight culture of E. coli strain BL21 (DE3) harboring the expression plasmid pFimC. Cells were induced at an OD₆₀₀ of 0.5 with isopropyl-β-D-thiogalactoside (IPTG) (1 mM), grown for 16 h at 25 °C and harvested. The bacteria were resuspended in 100 mM 50 mM Tris/HCl buffer (pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mg ml⁻¹ polyvinylammonium sulfate) and stirred for 2 h at 4 °C. After centrifugation, the supernatant (periplasmic extract) was dialyzed against 10 mM Tris/HCl at pH 8.0 and applied to a DE52 cellulose column (Whatman), which was then washed with the same buffer. The flowthrough was applied to a CM52 cellulose column (Whatman), and FimC was eluted with a linear NaCl gradient (0-500 mM) in 10 mM Tris/HCl, pH 8.0. Fractions containing pure FimC were pooled and dialyzed against H₂O. For production of uniformly ¹⁵N-labeled FimC, minimal medium containing (¹⁵NH₄)₂SO₄ (15 µmol l⁻¹) and unlabeled glucose (5 g l⁻¹) was used, and the protein was purified as described above. The yields of purified protein were 1.4 and 2.8 mg l⁻¹ of bacterial culture for ¹⁴N,¹⁵H-labeled FimC and ¹⁴N-labeled FimC, respectively.

Purification of FimH. E. coli HM12519 harboring the plasmid pFimH-FimC, which allows coexpression of FimH and FimC from a dicistronic operon under the control of the trc promoter, was grown at 30 °C in 1 L LB medium containing ampicillin (100 µg ml⁻¹). Protein expression was induced at an OD₆₀₀ of 1.7 with IPTG (1 mM). The bacteria were harvested 18 h after induction and subjected to periplasmic extraction as described above. The periplasmic extract was dialyzed against 10 mM MOPS/NaOH, pH 7.0 and applied to a DE52 cellulose column coupled to a CM52 cellulose column. The FimC–FimH complex was eluted from the CM52 column with a NaCl gradient (0-400 mM) and the fractions containing the pure FimC–FimH complex were pooled and dialyzed against 10 mM MOPS/NaOH, pH 7.0. The complex was then dissociated by addition of 9 M urea to a final concentration of 4 M urea. The solution was applied to a CM52 column equilibrated with 4 M urea/10 mM MOPS/NaOH pH 7.0 and the fractions containing pure FimH were collected and concentrated by ultrafiltration.

Protein concentrations. The concentrations of FimC and FimH were determined by their specific absorbance at 280 nm (at a concentration of 1 mg ml⁻¹ in a 1 cm cuvette) with values of 1.07 and 1.09, respectively.

Reconstitution of the complex between ¹⁴N,¹⁵H-labeled FimC and unlabeled FimH. Purified FimH (18 µM in 4.0 M urea, 10 mM MOPS/NaOH, pH 7.0) was diluted to a urea concentration of 0.48 M. Then 0.9 molar equivalents of ¹⁴N,¹⁵H-labeled FimC (0.4 mM in H₂O) were added and the solution was incubated for 15 min at 4 °C. The FimC–FimH complex was extensively dialyzed against H₂O and concentrated to 0.4 M. Excess FimH, aggregated during concentration, was removed by centrifugation. The pH was adjusted to 5.0 with HCl and the solution was filtered (pore size 0.2 µm) before the NMR measurements. The 1:1 stoichiometry of the complex was verified by analytical gel filtration on a Superdex 75 column (Pharmacia) in 50 mM sodium phosphate, pH 7.0. The complex between ¹⁴N-labeled FimC and unlabeled FimH was reconstituted with the same protocol, except that no excess of FimH over FimC was used. Formation of the two disulfide bonds in FimH was evidenced by the lack of free thiols after unfolding of FimH according to Elman's assay.

NMR spectroscopy. All NMR experiments were carried out at 38 °C and pH 5.0 on a Bruker DRX750 spectrometer. [¹⁴N,¹⁵H]-TROSY spectra were recorded as described with a sample of 0.4 mM ¹⁴N,¹⁵H-labeled FimC and a sample of 0.4 mM ¹⁴N,¹⁵H-labeled FimC complexed with unlabeled FimH. The total measurement time was 6 h for each experiment. In addition, conventional [¹⁴N,¹⁵H]-correlation spectra were measured with the above mentioned samples and with a sample containing uniformly ¹⁴N-labeled FimC complexed with unlabeled FimH. In all samples the solvent was 90% H₂O and 10% ²H₂O. All spectra were processed with the program PROSAD and analyzed with the program XEASY. For the preparation of the illustrations with molecular models we used the program MOLMOL.

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