



F4 fimbriae expressed by enterotoxigenic *Escherichia coli* are the major colonization factor associated with porcine post weaning disease or porcine colibacillosis, leading to severe economic losses in pig industry worldwide (Frydendahl, 2002). F4 fimbriae are polyadhesins of the major subunit FaeG (Bakker *et al.*, 1992a), assembled via the chaperone-usher (CU) pathway, a major pathway applied by Gram-negative bacteria to assemble a wide variety of morphologically very distinct adhesive structures on their surface (Thanassi *et al.*, 1998; Sauer *et al.*, 2004). Subunits of CU assembled pili, or pilins, share an incomplete immunoglobulin (Ig) like fold, lacking the seventh β -strand, G. All but the tip subunit also contain a 10 to 18 residue long *N*-terminal extension (Nte) that is not part of the Ig-like core (Sauer *et al.*, 1999; Choudhury *et al.*, 1999). Via a conserved donor strand complementation/exchange mechanism, these pilins are non-covalently linked to form highly stable polymers (Sauer *et al.*, 2004; Zavialov *et al.*, 2002; Capitani *et al.*, 2006; Remaut *et al.*, 2006; Erilov *et al.*, 2007; Puorger *et al.*, 2008).

Although the receptor binding by F4 fimbriae has been intensively studied, the identity of this receptor has not yet been elucidated (Francis *et al.*, 1999; Grange *et al.*, 2002), nor has the receptor binding site on FaeG. Three antigenic variants of F4 have been identified, F4_{ab}, F4_{ac} and F4_{ad}, showing different receptor binding specificity (Orskov *et al.*, 1964; Guinee and Jansen, 1979). Most probably all variants recognize a common carbohydrate core, with modifications to this core giving rise to variant specificity. One of the major goals of this PhD study was to unravel and structurally characterize the receptor binding by FaeG. To enable the expression of soluble, stable recombinant FaeG monomers, in the conformation they display in the F4 fimbriae, it was primordial to gain insight in the biogenesis of F4 fimbriae.

The first details of the structure of FaeG were obtained from a plant-expressed FaeG. In the absence of the assembly machinery, tobacco chloroplasts assemble FaeG to strand swapped dimers. This misfolding points out the importance of the chaperone, in this case FaeE, for proper pilin folding. Interestingly, the strand-swapping between strands A₁ and A₂ seen in the dimeric structure of the chloroplast-targeted FaeG, similarly occurred in the cytoplasm of *E. coli* when the subunits of the Dr fimbrial family were expressed in the absence of their cognate chaperone (Anderson *et al.*, 2004; Pettigrew *et al.*, 2004; Korotkova *et al.*, 2006). A role of the donor strand of the chaperone during pilin folding could thus be to keep the A₁ and A₂ strands retained within the Ig-core. The chaperone would thereby prevent the subunits from folding into an assembly-incompetent form prior to *in vivo* fimbrial assembly.

The unswapped model of the plant expressed FaeG indicated that residues 1 to 17 form the Nte involved in donor strand exchange (DSE). This was confirmed by the structure of a donor

strand complemented FaeG construct (FaeG_{ntd/dsc}), in which residues 2 to 17 of the Nte were covalently attached to the C-terminus of FaeG_{ntd}, via a DNKQ linker, in order to form a stable, monomeric FaeG construct. In FaeG_{ntd}, residues 5 to 21 were replaced by a His₆-tag to prevent polymerization. Upon complementing another FaeG pilin's fold, the side chains of residues Phe6, Val10, Ile12 and Ile16 of the Nte are buried in the P1, P2, P3 and P5 pockets in the hydrophobic groove on the surface of this FaeG molecule. Residues 1 to 5 of the Nte lay in a bend in the groove on the surface of FaeG, allowing insertion of Trp1 in an extra pocket in the groove (P*). The Nte of FaeG extends beyond the P1 to P5 residues forming the G strand that complements the fold of another FaeG pilin in the mature pilus. Accordingly, this hydrophobic groove also extends beyond the P1 to P5 pockets. Elongation of the Nte and the hydrophobic groove appears to be a general feature of major pilins since it has also been observed for PapA, SafA and Caf1M (Verger *et al.*, 2007; Remaut *et al.*, 2006; Zavialov *et al.*, 2003). This elongation is important for pilus formation and stability. Indeed, deletion of residues 1 to 4 of FaeG leads to decreased polymer stability as was determined by differential scanning calorimetry measurements. As for SafA, opening of the extra P* pocket in the groove on the surface of FaeG requires conformational changes coupled to DSE, allowing insertion of the anchoring P* residue necessary for completion of the DSE reaction (Remaut *et al.*, 2006).

As for other CU assembled structures, DSE stabilizes FaeG, as was shown both by structural details as by differential scanning calorimetry (DSC) measurements. DSC profiles of FaeE-FaeG_{ntd} and FaeG_{ntd/dsc} were irreversible and scan rate dependent, indicating that the thermal unfolding of FaeG is kinetically determined. Thus, the DSC profiles can be described by a two-state irreversible unfolding model (native → final state). The linear dependency of the melting temperature (T_m) on the scan rate allowed calculation of the activation energy (E_a) of the irreversible unfolding step. The increase of the both the T_m and E_a values of FaeG before and after DSE (FaeG_{ntd} in complex with FaeE and FaeG_{ntd/dsc}, respectively) shows that DSE indeed stabilizes FaeG. Stabilization of FaeG through DSE, alike for other CU assembled pilins, is a consequence of the collapse of the Ig fold upon replacement of the chaperone's donor strand with the Nte. Comparison of the structure of FaeG before and after DSE shows that the two sheets of the Ig-like fold indeed come closer towards each other. The better fit of the two sheets is confirmed by the increase in shape correlation statistic (Sc) for the two sheets from 0.61 to 0.73, indicating a better fit after donor strand exchange than before (Sc = 0 means no fit, while Sc = 1 is a perfect fit). Similarly, the two sheets in the Ig-like folds of

PapE and CafI show an increase of the Sc from 0.57 to 0.72 and 0.58 to 0.72, respectively (Zavialov *et al.*, 2003).

The crystal structure of the periplasmic chaperone involved in the biogenesis of F4 fimbriae, FaeE, shows a dimer formed by means of anti-parallel β -strand pairing of its pilin-interactive G₁ strands. This dimer was also observed in the crystal structures of PapD and SfaE (Hung *et al.*, 1999; Knight *et al.*, 2002). CafIM, the cognate chaperone of CafI, was shown to form tetramers involving the subunit binding interfaces. These tetramers were also present in solution (Zavialov and Knight, 2007). Di- or multimerization of pilin-free fimbrial chaperones was suggested to serve as a mechanism to shield the hydrophobic subunit binding regions (Hung *et al.*, 1999; Knight *et al.*, 2002; Zavialov and Knight, 2007). However, the pilin free form of FaeE is present predominantly as a monomer in solution. Oligomerization or self-capping of fimbrial chaperones is thus not an absolute requirement for FaeE stability in solution, nor is it a general mechanism to shield the pilin-interactive motif in free periplasmic chaperones. Oligomerization of the CafIM chaperone is correlated with the high β -aggregation propensity of its G₁ strand. Understandably, the presence of an excess of free periplasmic chaperone, conditional for its crystallization, would be rather unusual during pilus biogenesis, hence the need for self-capping and thus protection of free chaperone would be minor.

The structure of FaeG gives the first structural insight in the F4 receptor binding and thereby adds valuable information to the knowledge on receptor-binding by polyadhesins. Unlike for pilus systems which display a two domain adhesin (TDA) at their tip, containing a specialized receptor binding domain, single domain adhesins (SDAs) have to combine their structural function with their receptor-binding capacity. Little receptor-binding details are available to elucidate how SDAs manage this. Mapping of the residues proposed to be involved in F4 receptor binding (Jacobs *et al.*, 1987a; Jacobs *et al.*, 1987b; Bakker *et al.*, 1992b; Zhang *et al.*, 2009) and the hypervariable regions between the three F4 variants on the structure of FaeG, shows that FaeG combines its dual role by grafting a receptor binding domain on the Ig-like core. The recent NMR structure of the AfaE-III SDA in complex with the N-terminal domain of carcinoembryonic antigen (CEA) (Korotkova *et al.*, 2008), showing interaction through β -sheet packing between the Ig-cores of both proteins, indicates that grafting of an additional receptor-binding domain on the structural Ig-like core does not appear to be a general principle applied by SDAs to combine their structural and functional roles. Receptor-binding details of other SDAs for which the structures have been solved, such as CafI (Zavialov *et al.*,

2003) and SafA (Remaut *et al.*, 2006), are lacking. Additionally, the receptor for the F4 related F5 fimbriae has been identified (Kyogashima *et al.*, 1989) but no structural details are available yet for the FanC SDA.

Two independent glycan arrays indicated binding of FaeG to α - and β -linked glucooligosaccharides. Docking experiments for maltotetraose agree with the receptor-binding site indicated on the structure of FaeG. Two partially overlapping binding positions for maltotetraose were identified within this site. Binding of maltotetraose, maltopentaose, maltohexaose and maltoheptaose by FaeG was also confirmed using surface plasmon resonance measurement. However, the affinity of the monomeric FaeG constructs used in this study for these glycans is only in the millimolar range. This low affinity suggests the possible importance of multivalent receptor interactions by the F4 polyadhesin, as was shown for the glycogen binding by the SpuA and Pula pullulanases of *Streptococcus pneumoniae* and *S. pyogenes* (van Bueren *et al.*, 2007), or could be an effect of the three dimensional conformation in which the glycans are presented, as in the case of the starch-binding module SusD of *Bacteroides thetaiotaomicron* (Koropatkin *et al.*, 2008). Additionally, the low affinity of FaeG for glucooligosaccharides could be correlated with the fact that these glycans do not represent the physiological receptor for FaeG on pig intestines.

In summary, the major contribution of this study is the structural insight given in the receptor binding by F4 fimbriae. Further research using oligomeric FaeG constructs and different carbohydrate conformations should reveal more insights in the glucooligosaccharide-binding properties of F4 fimbriae. Furthermore these FaeG multimers will also be used in novel F4 receptor-identification strategies such as natural glycan arrays displaying glycan structures isolated from piglet intestinal cells, which are currently being developed in collaboration with Dr. Manfred Wuhrer at the Leiden University Medical Center.

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