Expression of bacterial adhesins in *E.coli*: From mapping of adhesive epitopes to structure

Jarna Tanskanen

Faculty of Science
Department of Biosciences
Division of General Microbiology
and
Graduate School in Microbiology
University of Helsinki

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Supervisors: Docent Benita Westerlund-Wikström
Department of Biosciences
Division of General Microbiology
University of Helsinki, Finland

Professor Timo Korhonen
Department of Biosciences
Division of General Microbiology
University of Helsinki, Finland

Reviewers: Docent Kristiina Takkinen
VTT Biotechnology, Biomolecules
Finland

Professor Dennis Bamford
Department of Biosciences
Division of Genetics
University of Helsinki, Finland

Opponent: Professor Per Klemm
Department of Microbiology
Technical University of Denmark
Lyngby, Denmark

Cover figure:
A stereo view of the surface of the GafD1-178 showing the bound GlcNAc
(courtesy of Michael Merckel)

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which in the text are referred to by their Roman numerals.

I **Westerlund-Wikström, B., J. Tanskanen, R. Virkola, J. Hacker, M. Lindberg, M. Skurnik and T. K. Korhonen**. 1997. Functional expression of adhesive peptides as fusions to *Escherichia coli* flagellin. Protein Eng. **10:** 1319-1326.

II **Tanskanen, J., T. K. Korhonen and B. Westerlund-Wikström**. 2000. Construction of a multihybrid display system: flagellar filaments carrying two foreign adhesive peptides. Appl. Environ. Microbiol. **66:** 4152-4156.

III **Tanskanen, J., S. Saarela, S. Tankka. N. Kalkkinen, M. Rhen, T. K. Korhonen and B. Westerlund-Wikström**. 2001. The *gaf* gene cluster of *Escherichia coli* expresses a full-size and a truncated soluble adhesin protein. J. Bacteriol. **183:** 512-519.

IV **Merckel, M. C., J. Tanskanen, S. Edelman, B. Westerlund-Wikström, T. K. Korhonen and A. Goldman**. 2002. The crystal structure of *Escherichia coli* G-fimbrial lectin receptor binding domain GafD178 in complex with N-acetyl-D-glucosamine. (Manuscript)

SUMMARY

A multivalent flagella display system on *Escherichia coli* cell surface was developed. The system, which is based on a genetic fusion to the variable region in *fliC*, tolerates large inserts and is suitable for expression of bacterial adhesive epitopes. The model peptide, the D repeats of *Staphylococcus aureus* FnBPA, as well as the YadA peptide from *Yersinia enterocolitica* O3, were expressed in a functional form. The D repeats fused to FliC bound to soluble as well as cellular fibronectin in a dose-dependent, saturable manner. The binding was most efficient with the flagella expressing all three D repeats. By analyzing various YadA fragments fused to FliC, the collagen-binding region was localized to the residues 84-385 in YadA. This suggests that the collagen-binding region in YadA is long and probably non-linear. The adhesin-FliC fusions encoded on compatible plasmids were expressed simultaneously in the same flagellar filament, creating a bifunctional display system. Chimeric flagella were used to raise anti-adhesive antibodies that inhibited the adherence of *S.aureus* to fibronectin. Flagellin seems quite permissive for inserts that differ in size and chemical properties, and our results indicate that the flagella display can be succesfully used in receptor-ligand studies.

The GafD fimbrial lectin was isolated from bacterial periplasm in a truncated form, ΔGafD (178 N-terminal amino acids). ΔGafD was also detected in the periplasm of the wild-type *E.coli* strain from which the *gaf* gene cluster originally was cloned. The truncate was not detected in G-fimbrial filaments, which indicates that ΔGafD is not competent for assembly. ΔGafD was completely soluble and expressed the full receptor-binding specificity of the G fimbriae of bovine septicaemic *E.coli*. The structure of the adhesin domain GafD1-178 was determined to 1.7Å resolution in the presence of the receptor *N*-acetyl-D-glucosamine (GlcNAc). The overall topology is similar to the β-barrel jelly roll-like fold earlier reported for the fimbrial lectins FimH and PapG of *E.coli*. GafD1-178 consists of 16 β–strands and one 3/10 helix. The receptor-binding site is on one side of the molecule and forms an extended cleft consisting of the C-terminal end of β-strand 6, the 3/10 helix and β-strands 7, 8 and 9 that interact with GlcNAc mainly via hydrogen bonds from side chain as well as main chain atoms. Ala43-Asn44, Ser116-Thr117 form the carbohydrate acetamide specificity pocket, while Asp88 confers tight binding and Trp109 appears to position the receptor. A disulphide bond between the β–strands 3 and 8 probably stabilizes the binding site. GafD1-178, FimH and PapG share similar β-barrel folds but differ in receptor specificity and disulphide bond patterns. The resolved GafD1-178 structure expands our knowledge on the adhesive mechanisms as well as on the evolution of fimbrial lectins.

1. INTRODUCTION

In nature, bacteria commonly live attached to surfaces. Bacterial adhesion to tissues is often the first and essential step in colonization of host surfaces by indigenous as well as pathogenic bacteria. To overcome mechanical defenses of the host, bacteria need to adhere to epithelial surfaces or to underlying tissue. Specific recognition proteins, adhesins, mediate bacterial adhesion by binding to receptor molecules on tissues. Bacterial adhesins may also interfere with the immune system, trigger signaling pathways in the bacterium or the host, assist delivery of effector molecules into host cells, or promote bacterial invasion (reviewed in Finlay and Falkow, 1997; Mulvey, 2002). It is therefore not surprising that adhesins contribute to bacterial virulence (Hultgren *et al.*, 1996) and that research on the molecular mechanisms of adhesin-receptor interactions has received attention.

Nearly all characterized bacterial adhesins are surface proteins, and bacteria have developed several structural frameworks for presenting their adhesive peptides. The net negative charge of both bacterial and tissue cell surfaces repulse adhesion (Donnenberg, 2000). Therefore, in order to achieve binding, the functional part of the adhesin is often located distal to the bacterial surface in varying protein architectures, typically in hair-like appendages called fimbriae. Bacterial adhesins vary in their affinity, and often the low affinity of individual epitopes is strengthened by expression in multiple copies on bacterial surface. Structural organization of adhesin molecules is thus linked to function.

1.1. The diversity of bacterial adhesins

The most common adhesive organelle found on enteric bacteria is the fimbria. For example, type 1 fimbriae are produced by almost all species and isolates of the family *Enterobacteriaceae*. Fimbriae are polymeric protein organelles ranging from 2 to 7 nm in width and protruding 0.2 to 20 μm from the bacterial cell surface (reviewed in Hultgren *et al.*, 1996; Klemm and Schembri, 2000; Mulvey, 2002). The fimbrial filament is composed of hundreds of copies of the helically arranged major subunit protein, fimbrillin, and a few copies of minor fimbrial proteins, which in most cases include the adhesin. The adhesin subunit can be located in a thin tip-fibrillum at the filament tip as seen in the high-resolution immunoelectron micrographs of PapG of the P fimbriae (Kuehn *et al.*, 1992) and also along the main fimbrial filament as shown in the type 1 fimbriae (Abraham *et al.*, 1987; 1988; Krogfelt *et al.*, 1990). The fimbrillin itself can also be the adhesin, as exemplified by FaeG and FanC of the K88 and the K99 fimbriae of enterotoxigenic *E.coli* (Erickson *et al.*, 1992; Smit *et al.*, 1984) or DraE of the Dr fimbriae of uropathogenic *E.coli* (Nowicki *et al.*, 1989).

Bacterial adhesion can also be mediated by integral membrane proteins such as OmpA of *E.coli* (Prasadarao *et al.*, 1996), and proteins that form a polymeric layer on the bacterial surface, such as YadA of *Yersinia enterocolitica,* which covers the bacterium by forming a fibrillar layer on top of the cell wall (Hoiczyk *et al.*, 2000). Paracrystalline surface protein arrays called S-layers (reviewed in Sára and Sleytr, 2000), such as the CbsA of *Lactobacillus crispatus* (Toba *et al.*, 1995) and SlpA of *Lactobacillus brevis* (Hynönen *et al.*, 2002) as well as the A-layer of *Aeromonas salmonicida* (Chu *et al.*, 1991), confer bacteria the ability to bind to eukaryotic molecules. Enteric bacteria also possess adhesins that remain associated to the cell wall via their

membrane-integrated, translocator domains. AIDA-1, an autotransporter of enteropathogenic *E.coli*, confers diffuse adherence to cultured mammalian cells via its amino-terminal (N-terminal) α-domain (Benz and Schmidt, 1989; 1992).

1.2. Bacterial adhesins mediate attachment to a variety of targets

Adhesins direct the bacteria to a specific location in the host. The specificity of the adhesin-receptor interaction determines the host and the tissue tropism but other factors, such as nutritional requirements, also affect colonization at specific niches. For the bacteria, it may be beneficial to either possess multifunctional organelles or an array of adhesins which vary with time of expression or in receptor specificities, both alternatives enhance recognition of different surfaces. Upon entering the host, bacteria normally confront first epithelial surfaces and many of the recognized bacterial adhesins indeed bind to epithelial cells. Interestingly, rather than utilizing a host receptor, enteropathogenic *E.coli* (EPEC) secrete a receptor protein, translocated intimin receptor (Tir), that is transferred onto the epithelial cells where it binds the EPEC adhesin, intimin (reviewed in Frankel *et al.*, 1998). Various bacteria initiate infection through damaged epithelium, such as in wounds, and thus face subepithelial tissue structures, the extracellular matrix (ECM). Adhesion to matrix proteins facilitates colonization and invasion of bacteria, suggesting that this interaction has a role in pathogenicity (reviewed in Westerlund and Korhonen, 1993). Adhesion to the epithelium as well as to the ECM can be manifested by the same adhesin or even by the same functional domain in the adhesive protein, which exemplifies the multifunctional nature of bacterial adhesins.

Many bacterial adhesins recognize mammalian cell surface carbohydrates and thereby function as lectins. The best characterized bacterial lectins are the mannose-specific FimH of the type 1 fimbriae (Krogfelt *et al.*, 1990) and the digalactoside-specific PapG of the P fimbriae (Lindberg *et al.*, 1984; Lund *et al.*, 1987). Also, noncarbohydrate regions in proteins can be targets for bacterial adhesins. The binding of DraE of the Dr fimbriae to the Dr^a blood group antigen on decay-accelerating factor (Nowicki *et al.*, 1988; Van Loy *et al.*, 2002; 2002a) and to type IV collagen (Westerlund *et al.*, 1989), as well as the binding by the MrkD protein of the type 3 fimbriae of *Klebsiella pneumoniae* to type V collagen (Tarkkanen *et al.*, 1990) are examples of protein-protein interactions in bacterial adhesins. Furthermore, the minor proteins of P fimbriae, PapE and PapF, mediate adhesion to immobilized fibronectin (Westerlund *et al.*, 1989a; 1991) and variants of FimH recognize fibronectin (Sokurenko *et al.*, 1994) or collagen (Pouttu *et al.*, 1999) by protein-protein interaction. These fimbriae are multifunctional in terms of the adhesion mechanisms and target molecules. Certain bacterial adhesins, such as the fibronectin-binding protein A (FnBPA) of *Staphylococcus aureus*, bind circulating host proteins onto bacterial surface, which enhances bacterial adhesion or invasion through a bridging mechanism (Massey *et al.*, 2001).

By understanding bacterial adherence mechanisms, our comprehension of pathogenic processes expands and hopefully leads to development of novel antiadhesive measures. Several studies indicate that anti-adhesin vaccines provide protection against bacterial infections (reviewed in Klemm and Schembri, 2000). The use of FimH as an immunogen in a murine cystitis model protected against experimental infection with uropathogenic *E.coli* (Langermann *et al.*, 1997; Thankavel *et al.*, 1997). Furthermore, passive systemic administration of anti-FimH antiserum

inhibited more effectively *E.coli* adhesion to bladder epithelial cells than did the antiserum against the type 1 fimbriae (Langermann *et al.*, 1997), which has further encouraged purification and structure determination of bacterial adhesins.

A multitude of adhesins with defined receptor specificities have been described in *E.coli*. Yet, only a handful of atomic structures of bacterial adhesins have been reported so far. In wild-type bacteria, adhesion proteins are normally in numbers too low to allow purification in the amounts needed for structure determination. The instability of overexpressed recombinant adhesion proteins**,** especially the fimbrial adhesins, further complicates purification. Bacterial adhesins recognize their receptors often with low affinity, a problem that interferes with binding studies using recombinant adhesins or peptides thereof. Affinity may further decrease when the adhesin domains are expressed as separate fragments (Signäs *et al.*, 1989; Huff *et al.*, 1994; Rich *et al.*, 1998). To overcome these difficulties, fusion peptides presented in numerous copies on the surface of a bacterium or a bacteriophage have been produced; these systems often increase the avidity of the binding. Surface display systems provide means to characterize adhesin-receptor interactions and to identify novel bacterial ligands.

2. SURFACE DISPLAY OF BACTERIAL ADHESIVE PEPTIDES

Surface display systems enable the study of adhesive peptides on the surfaces of viruses or cells. These biological hosts provide a platform for the expressed molecule and harbor the nucleotide sequence that encodes the displayed peptide. As protein display systems generally use the secretion machinery of the host, the peptide to be displayed has to be compatible with the protein export system being utilized. The foreign peptide is genetically fused to a carrier protein which facilitates translocation and anchors the peptide to the surface of the host. Surface expression has been used to identify receptor-binding epitopes of bacterial adhesins (Jacobsson and Frykberg, 1995; Zhang *et al.*, 1998; Lång *et al.*, 2000; Beckmann *et al.*, 2002), for affinity selection of peptides with desired properties or for development of novel biocatalysts or adsorbents (reviewed in Georgiou *et al.*, 1997; Ståhl and Uhlén, 1997; Klemm and Schembri, 2000b; Benhar, 2001). To date, antibody engineering and vaccine development have been the most common applications of surface display techniques.

An optimal carrier protein does not disrupt the conformation of the displayed peptide and accepts epitopes of various size without severely damaging its own translocation or conformation. The choice of carrier depends on the desired application: the valency of the display system affects affinity, the secretion pathway of the carrier may affect folding and thereby function of the insert, and the need for either purified chimeric proteins or whole cells determines the type of display system one wishes to use. The present display systems have a broad host range and repertoire of carriers, but problems remain with the size limitation of the displayed protein, its correct localization on the host surface, as well as with the instability of bacterial membrane or viral coat caused by overproduction of chimeric proteins. A few surface display systems have proven successful in identification of adhesive epitopes in bacterial adhesins. However, the present emphasis on surface display is still in the development of the systems, and especially the adhesion proteins of Gram-negative bacteria with their disulphide bonds and low affinity have proven demanding subjects for surface display.

2.1. Filamentous phage display

Filamentous phage display technology is based on genetic fusion of foreign peptides to the structural proteins of phage M13 or its derivatives (reviewed in Rodi and Makowski, 1999). The recombinant coat protein is incorporated into phage particles that are assembled in the periplasm of *E.coli*. Two structural proteins of the phage, the pIII and the pVIII protein, are used as carriers for display. Three to five copies of the minor coat protein pIII, needed for phage infectivity, reside at the end of the filamentous phage particle. Infectivity of the phage can be maintained by insertions into the N-terminus of the pIII or by using a phagemid system, where helper phage provides intact pIII (Lubkowski *et al.*, 1998). The latter system allows the expression of larger inserts such as antibody Fab fragments (Barbas *et al.*, 1991). The atomic resolution structure of the N-terminal domains of the pIII is resolved (Lubkowski *et al.*, 1998) and provides detailed information on suitable insertion sites for display purposes. Multivalent display can be accomplished in the major coat protein, pVIII. The pVIII protein tolerates only short inserts without loss of the phage stability (Iannolo *et al.*, 1995). Mosaic phage particles expressing wild-type pVIII as well as recombinant pVIII can be successfully used to express larger inserts (Smith, 1993). Recombinant phage particles carrying the desired inserts can be enriched by biopanning i.e. repeated binding, washing and elution steps with subsequent amplification of the eluted phage in *E.coli* (Parmley and Smith, 1988).

Phage display has most commonly been used for antibody engineering, i.e. the selection of protein variants with novel binding properties, higher binding affinity or specificity (reviewed in Hoogenboom *et al.*, 1998). Recently, various receptor-binding epitopes of bacterial adhesins have been identified using a shotgun phage display library, in which the chromosomal or adhesin gene fragments are fused to *pIII* or *pVIII* in a phagemid vector which contains the origin of replication from the plasmid ColE1 and phage M13 (Table 1). Rosander and colleagues (2002) searched for extracellular proteins by fusing DNA fragments of *S. aureus* to a *pIII*-vector devoid of a signal sequence. This way only inserts containing a signal sequence are exported to the inner membrane of *E.coli* and assembled into phage particles. This strategy allows the identification of novel extracellular proteins irrespective of their function.

Table 1. Examples of receptor-binding epitopes of bacterial adhesins identified using a shotgun phage display technique

Source	Fusion (pIII / pVIII)	Adhesin	Target	Binding domain (aa)	Reference
Staphylococcus aureus	III	Sbi	human IgG	52	Jacobsson and Frykberg, 1995; Zhang *et al.*, 1998
Staphylococcus aureus	VIII	Sbi	β_2-glycoprotein I	58	Zhang *et al.*, 1999
Staphylococcus epidermidis	VIII	Fbe	fibrinogen	331	Nilsson *et al.*, 1998
Streptococcus dysgalactiae	VIII	DemA	fibrinogen	266	Vasi *et al.*, 2000
Streptococcus equi subsp. *zooepidemicus*	III	FNZ	fibronectin	57	Lindmark *et al.*, 1996
Streptococcus agalactiae	III	ScpB	fibronectin	112	Beckmann *et al.*, 2002
Staphylococcus aureus	VIII	vWbp	von Willebrand factor	26	Bjerketorp *et al.*, 2002

2.2. Surface display on Gram-negative bacteria

A large number of bacterial surface proteins have been employed as carriers of foreign peptides (Table 2). The multitude of carriers and hosts give flexibility to bacterial surface display which has been used to generate recombinant vaccines, reagents in diagnostics, biocatalysts or bioadsorbents, platforms for the screening of peptide libraries (reviewed in Georgiou *et al.*, 1997; Ståhl and Uhlén, 1997), and lately also tools in determining the receptor-binding epitopes of bacterial adhesins.

Outer membrane proteins (Omps) of Gram-negative bacteria can serve as carriers in whole cell display formats (reviewed in Lång, 2000). The inserts in surface loops of Omps have in most cases been short, i.e. less than 66 amino acids, but longer peptides up to 250 residues have been successfully displayed in LamB, OmpS and FhuA (Steidler *et al.*, 1993; Lång *et al.*, 2000; Etz *et al.*, 2001). Autotransporters translocate from the bacterial periplasm via the autotransporter domain β, which forms a β-barrel in the outer membrane and assists the passage of the N-terminal α–domain to the cell exterior. The α domain stays attached to the surface and can be released by autocatalytic action (Suhr *et al.*, 1996; Konieczny *et al.*, 2001). Autotransporters are able to display large polypeptides in functional form (Suzuki *et al.*, 1995; Lattemann *et al.*, 2000; Kjaergaard *et al.*, 2002) and expression of disulphide-bonded peptides present in the inserts is dependent on the carriers as well as on the displayed peptide (Klauser *et al.*, 1992; Kjaergaard *et al.*, 2002). Heterologous peptides can be displayed on fimbrial filaments either as a fusion to the major or minor subunit, which provides a choice between a monovalent or a multivalent display (reviewed in Klemm and Schembri, 2000). The displayed inserts have been rather short as biogenesis of the fimbrial filament is disturbed by large inserts in the fimbrial subunits. In general, the position of the insert to be displayed requires careful consideration since several protein-protein interactions occur during assembly of the complex organelle. Fimbrial display

has been utilized in antigen presentation and in design of bioadsorbents (Table 2).

Gram-negative surface display platforms are anchored to the outer membrane, and the export of recombinant proteins occurs through inner and outer membranes except for flagella, which transports peptides directly from the cytoplasm. Hoischen and colleaques (2002) reported a novel surface display system which uses enterobacterial cells lacking the outer membrane. Staphylokinase (Sak) was fused into three integral inner membrane proteins, i.e. lactose permease, preprotein translocase of *E.coli* or CcmA of *Proteus mirabilis.* Sak was localized outside the cell and activated plasminogen, which demonstrated that the insert was functional. In contrast to normal bacteria, these protoplast-type cells lack periplasmic proteolytic activities.

Table 2. Examples of surface display systems in *E.coli*.
(modified from Klemm and Schembri, 2000b; Westerlund-Wikström, 2000)

Carrier	Expressed insert	Size of the epitope	Comments	Reference
Outer membrane proteins				
LamB	SpA domains	232 aa	functionality retained	Steidler *et al.*, 1993
OmpS	D repeats of FnBPA	115 aa	adhesion to soluble and immobilized fibronectin	Lång *et al,*. 2000
	PapG of P fimbriae	53-186 aa	aa 23-31 of PapG critical for binding	Lång *et al,*. 2000
Autotransporters				
Ag43	FimH	156 aa	mannose-binding retained	Kjaergaard *et al.*, 2002
Fimbriae				
FimA of type 1 fimbria	CtxB	34 aa	antibodies against chimeric fimbria recognized natural CtxB	Stenteberg-Olesen *et al.*, 1997
FimH of type 1 fimbria	preS2 of hepatitis B antigen, CtxB	56 aa 15 aa	FimH225 permissive site, FimH functional	Pallesen *et al.*, 1995
	random peptide library	26 aa	Zn^{2+} binding epitopes in a CtxB loop, FimH functional	Kjaergaard *et al.*, 2001
FasA of 987P fimbria	HSV-1 glycoprotein D, TGEV S	9aa 10aa	epitope recognized by antibodies, FasG functional	Rani *et al.*, 1999
FaeG of K88 fimbria	*Neisseria gonorrhoeae* pilin epitope	11 aa	insertion reduced the level of fimbriation	Bakker *et al.*, 1990
PapA of P fimbria	SpA domain A	58 aa	IgG-binding fimbria	Steidler *et al.*, 1993
Flagella				
FliC of *E.coli*	hen egg-white lysozyme epitope	11 aa	epitope recognition by antibodies, no immune response against chimeric flagella in guinea pigs	Kuwajima *et al.*, 1988
	random peptide library fused to thioredoxin	138 aa	conformationally constrained epitopes	Lu *et al.*, 1995
	fragments of SlpA of *Lactobacillus brevis*	61-272	81-residue N-terminal fragment of SlpA binds to human epithelium	Hynönen *et al.*, 2002

Abbreviations: SpA, Protein A of *Staphylococcus aureus*; FnBPA, fibronectin binding protein A; CtxB, cholera toxin B subunit; HSV-1, herpes simplex virus type 1; TGEV S, transmissible gastroenteritis virus spike protein; SlpA, S-layer protein A.

2.2.1. Flagella display

Flagella display is based on genetic fusion of foreign peptides into a surface-exposed, dispensable region of the flagellin FliC, the major subunit of the flagellar filament (reviewed in Westerlund-Wikström, 2000). The flagellar filament is composed of several thousand copies of FliC, a tip-associated FliD capping protein, and a few copies of FlgL and FlgK proteins that connect the filament to the hook structure composed of the FlgE protein (Figure 1). The hook docks the flagellum to the basal body in the outer membrane (reviewed in Macnab, 1996). The N- and carboxy-termini (C-termini) of FliC are highly conserved in eubacteria, whereas the central region of the flagellin is variable and confers antigenic heterogeneity (H antigenicity) to the flagella of *E.coli* and *Salmonella*. Kuwajima (1988b) has shown that deletion of 187 amino acids in the variable region of *E.coli* FliC does not affect flagellar assembly or motility. This discovery led to the flagella display system, which is based on *in trans* complementation of a chromosomal mutation in *fliC* in a nonflagellated *E.coli* strain and allows the expression of thousands of hybrid flagellins along the flagellar filament. Flagella display was first applied to vaccine development in attenuated *Salmonella*, and the use of chimeric flagella indeed induced immune response against the insert epitope (McEwen *et al.*, 1992; Levi and Arnon, 1996).

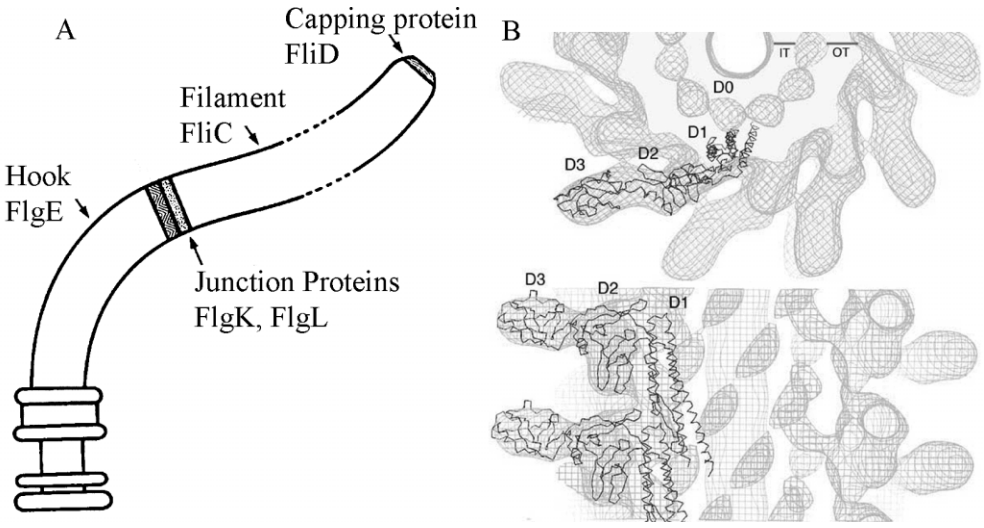

Figure 1. Bacterial flagellum. (A) Schematic presentation of a flagellar filament (Ikeda *et al*., 1996)**.** (B) Atomic model of a flagellar monomer docked into an electron density map (Samatey *et al*., 2001). Top panel, a cross-section view; bottom panel, a side view. FliC monomer is divided into four domains (D0-D3) of which the central part of the FliC (here as D3) forms the exterior of the flagellar filament. Fig.1A is reprinted by permission from Journal of Molecular Biology (Ikeda *et al.*, 1996), copyright (2002) Elsevier Science. Fig.1B is reprinted by permission from Nature (Samatey *et al*., 2001), copyright (2002) Macmillan Publishers Ltd.

3. BACTERIAL ADHESION PROTEINS WITH RESOLVED STRUCTURES

To date, few high resolution structures of bacterial adhesins or adhesive domains have been determined by X-ray crystallography or nuclear magnetic resonance spectroscopy (NMR) (Parge *et al.*, 1995; Symersky *et al.*, 1997; Pautsch and Schulz, 1998; Choudhury *et al.*, 1999; Hamburger *et al.*, 1999; Kelly *et al.*, 1999; Batchelor *et al.*, 2000; Hazes *et al.*, 2000; Luo *et al.*, 2000; Dodson *et al.*, 2001; Keizer *et al.*, 2001; Suh *et al.*, 2001; Sung *et al.*, 2001; Hung *et al.*, 2002; Prince *et al.*, 2002; Troffer-Charlier *et al.*, 2002). The resolved structures give insight into the adhesive mechanisms, the biogenesis and the evolution of adhesive organelles. With accumulating structural data, structural modelling can be applied to identify adhesive domains in homologous proteins. Furthermore, adhesins with similar binding properties can be found in data banks using a characterized domain as a template. The resolved binding epitopes in bacterial adhesins are conformational and can recognize overall receptor structures , an example is collagen recognition by Cna of *S.aureus* (Symersky *et al.*, 1997); or the epitope may mainly consist of main-chain atoms in a conserved conformation, which retains receptor specificity but allows antigenic variation (Hazes *et al.*, 2000).

3.1. Jelly roll motifs and immunoglobulin folds in fimbrial adhesins

Antiparallel β-barrel structures can form jelly roll folds, in which the β-strands of the polypeptide are wrapped around a barrel core (Branden and Tooze, 1991). Jelly roll motif is found in eukaryotic carbohydrate-binding, viral coat as well as in bacterial adhesive proteins.

3.1.1. FimH

Type 1 fimbriae bind to α-D-mannosides on mammalian tissue surfaces and confer colonization of commensal and pathogenic bacteria to various sites in the host, such as the intestine or the urinary tract (Bloch *et al.*, 1992; Connell *et al.*, 1996). Mannosyls are abundant in mammalian glycoproteins and type 1 fimbriae of *E.coli* interact with a multitude of surface-associated as well as secreted glycoproteins, such as bladder epithelial proteins, uroplakins (Wu *et al.*,1996; Mulvey *et al.*, 1998; Min *et al.*, 2002), laminin (Kukkonen *et al.*, 1993) as well as the secreted Tamm-Horsfall glycoprotein found in urine (Parkkinen *et al.*, 1988).

The first high-resolution structure of a fimbrial adhesin of *E.coli* was that of the chaperone-adhesin complex FimC-FimH of the type 1 fimbria (Choudhury *et al.*, 1999). The structure helped to obtain a model for fimbriae biogenesis as well as fimbrial lectin interaction with the receptor molecule. Later, Hung and coworkers (2002) defined the receptor-binding region of FimH in complex with D-mannose, and the crystal structure was in agreement with Choudhury and coworkers (1999). FimH has two domains connected by a short linker (Figure 2a). The N-terminal receptor-binding domain is a 11-stranded elongated β-barrel with a jelly roll-like fold. The fold begins with a short β hairpin that is not part of the jelly roll (Choudhury *et al.*, 1999) and the 11th strand breaks the jelly roll topology by going between the 3rd and 10th strands. The

receptor-binding pocket is situated at the tip of the adhesive domain and the residues in the pocket were found invariant in FimHs from 200 uropathogenic *E.coli* isolates (Hung *et al.*, 2002). The mannose-binding pocket is deep, negatively charged and surrounded by hydrophobic residues. The residues that interact with the carbohydrate are at the ends of β-strands or in the loops extending from them, except for Gln133 which is located within the strand 10 (Figure 2b). FimH and other fimbrial adhesins contain cysteine residues in the adhesive domain that form a disulphide bond important for binding activity (Carnoy and Moseley, 1997). The mannose-binding pocket of FimH contains one of the cysteines (Cys44) probably forming a disulphide bond within the adhesive domain.

The biogenesis of many fimbrial filaments follow the chaperone-usher pathway, which is used in assembly of over 30 fimbrial and non-fimbrial structures (reviewed in Sauer *et al.*, 2000; Thanassi and Hultgren, 2000; Schilling *et al.*, 2001), including the well-characterized type 1 and P fimbriae. The subunits of the type 1 fimbrial filament are exported to the periplasm via the general secretory pathway. The C-terminal fimbrillin-binding domain in FimH has an immunoglobulin-like fold that lacks the 7^{th} β-strand present in the canonical immunoglobulin folds (Choudhury *et al.*, 1999). The lack of this strand exposes a hydrophobic region which interacts with the FimC chaperone (Figure 2c). Fimbrial chaperones facilitate the folding of fimbrial subunits and prevent premature interactions with other subunits in the periplasm. A chaperone-subunit complex is delivered to the outer membrane usher FimD which forms a channel to allow the passage of fimbrial subunits. FimD drives the fimbrial assembly starting from the distal subunit until the filament extension is completed by a terminator protein. Fimbrial assembly is thought to proceed by a donor strand exchange mechanism (Choudhury *et al.*, 1999; Sauer *et al.*, 1999): The usher forms the assembly platform where the N-terminal extension of an incoming subunit displaces the chaperone G_1 strand. The immunoglobulin fold of every subunit is thus complemented by the N-terminal motif of its neighboring subunit.

3.1.2. PapG

The crystal and solution structures of the PapGII adhesin domain (Dodson *et al.*, 2001; Sung *et al.*, 2001) with the receptors globoside (GalNAcβ1-3Galα1-4Galβ1-4GlcCer) or galabiose (Galα1-4Gal) give important clues on fimbrial interaction with the receptor. The structure of the receptor-binding region PapG1-196 was resolved with and without globoside (GbO4) (Dodson *et al.*, 2001). The overall fold is an elongated jelly roll-like motif with two subdomains, a β barrel and an N-terminal receptor-binding subdomain (Figure 3a). The carbohydrate-recognition site (Figure 3a and b) resides on one side of the adhesin and forms a shallow pocket consisting of β-strands, an α-helix and a loop connecting a β-strand to the α-helix. The NMR structure of the PapG demonstrated the presence of a disulphide bond (Cys 44 and Cys 118) in the upper half of the binding domain (Sung *et al.*, 2001), which could stabilize two strands that form part of the binding site. The conformation of the PapG receptor site does not change upon globoside binding. The location of PapG at the tip of the flexible tip-fibrillum in P fimbria (Lindberg *et al.*, 1987; Kuehn *et al.*, 1992), the position of the binding site and the presence of charged residues nearby (Dodson *et al.*, 2001; Sung *et al.*, 2001), as well as the structure of digalactoside-ceramide (Pascher *et al.*, 1992) suggest a model for fimbria-uroepithelium interaction, where the binding domain in the tip fibrillum of P fimbria is oriented parallel to the membrane (Dodson *et al.*, 2001).

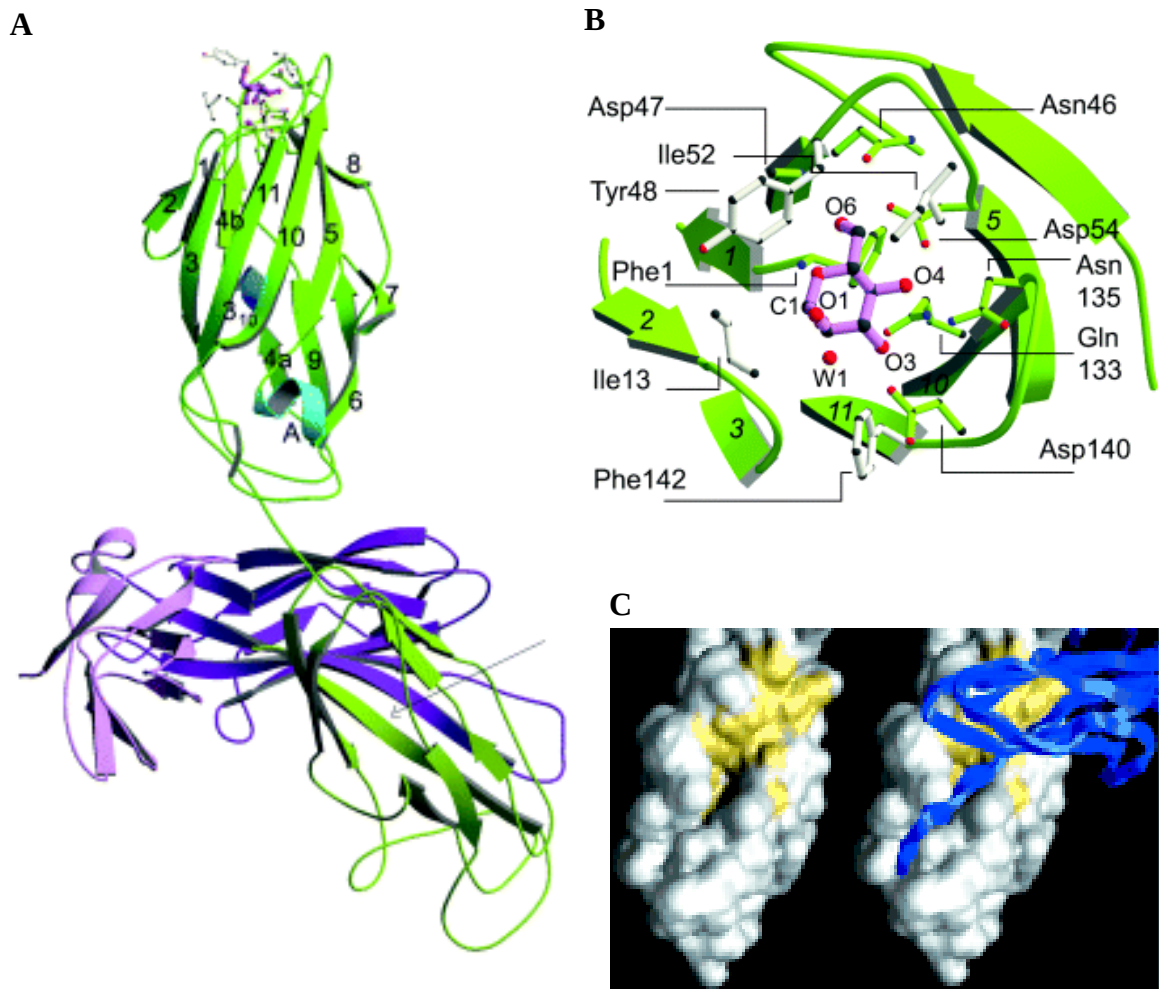

Figure 2. FimH structure. (A) A ribbon diagram of FimH-FimC complex bound to α-D-mannose (Hung *et al*., 2002). The receptor-binding domain in the upper part of the FimH comprises residues 1-158. The D-mannose (pink) binds to the tip of the domain. The fimbrillin domain interacts with FimC (purple and pink). FimC complements the missing strand in FimH (arrow). (B) The receptor-binding site (Hung *et al*., 2002). FimH residues that comprise the mannose-binding pocket are shown in green and the residues that form the hydrophobic ridge are in white, D-mannose is shown in pink. (C) A stereo view of the surface of the FimH fimbrillin domain showing the exposed hydrophobic core (Choudhury *et al*., 1999). Hydrophobic residues that are solvent exposed upon removal of the chaperone are shown in yellow, FimC (blue) complements the immunoglobulin-like fold in FimH. Fig. 2A and 2B are reprinted by permission from Molecular Microbiology (Hung *et al*., 2002), copyright (2002) Blackwell Publishing. Fig. 2C is reprinted by permission from Science (Choudhury *et al*., 1999), copyright (2002) American Association for the Advancement of Science.

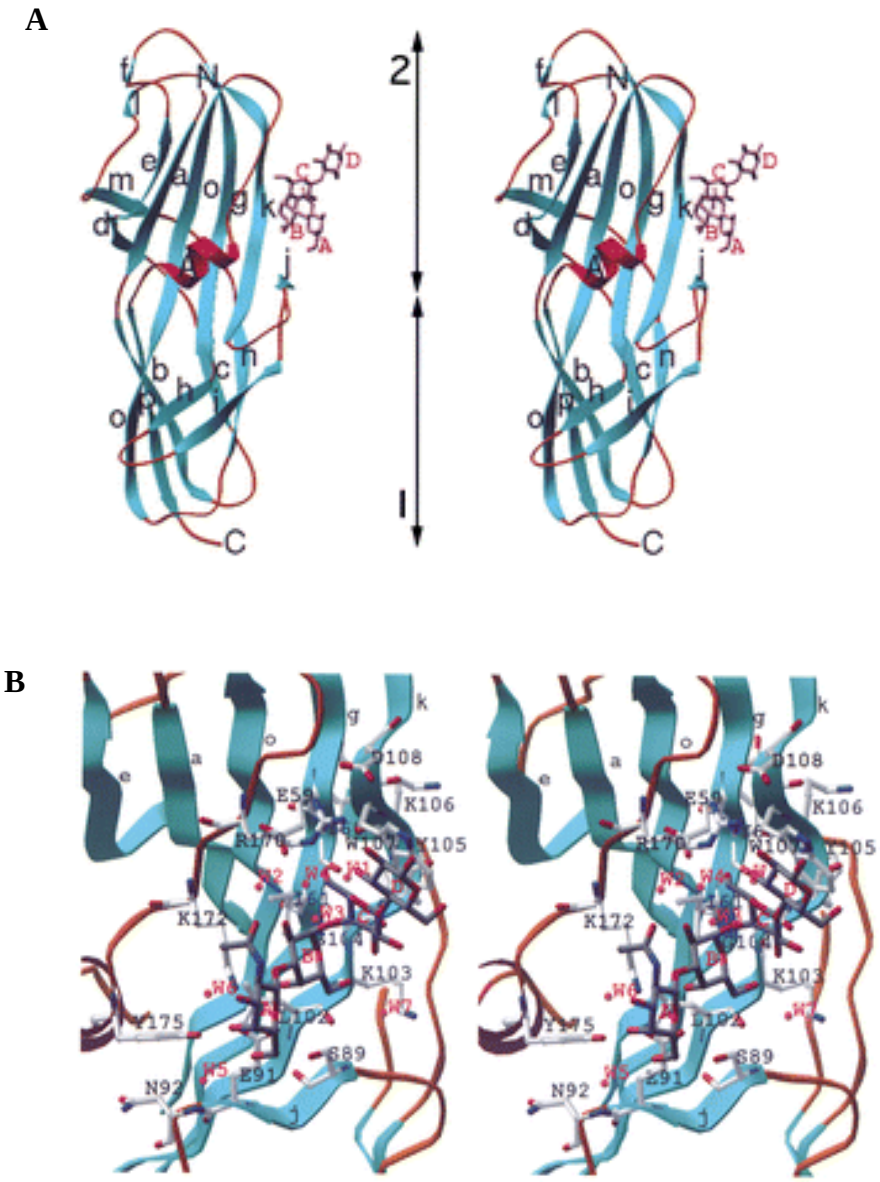

Figure 3. A stereo view of PapGII 1-196 bound to GbO4 (Dodson *et al*., 2001). (A) Structure of the PapGII 1-196: β strands are in cyan, α helix is in red and loops and coils are shown in orange. GbO4 binds to one side of PapG and is shown in ball-and-stick presentation. Sugar residues are indicated: A, GalNAc; B and C, Galα1-4Gal; C, Glc. (B) The receptor-binding site. Residues in the protein are in ball-and stick presentation, water molecules interacting with PapG and the globoside are indicated in magenta and labelled W1 to W7. Reprinted by permission from Cell (Dodson *et al*., 2001), copyright (2002) Elsevier Science.

3.2. Jelly roll folds in Gram-positive adhesins

S. aureus Cna mediates collagen binding (Patti *et al.*, 1992) and is an important virulence factor in septic arthritis (Switalski *et al.*, 1993; Patti *et al.*, 1994) and keratitis (Rhem *et al.*, 2000). Cna is composed of a 55 kilodalton (kDa) nonrepetitive collagen-binding domain A (Patti *et al.*, 1993; 1995), a B domain consisting of one to four repeats (Gillaspy *et al.*, 1997), and a cell wall anchor, a transmembrane segment, and a short positively charged cytoplasmic tail. Symersky and colleagues (1997) crystallized and determined the structure of the 19 kDa collagen binding part of the A domain. This polypeptide folds as a jelly roll and is composed of two β-sheets and two short α-helices. The groove on the first β-sheet forms the binding site for collagen, as suggested by a computer modelling with collagen fragments and analyses of single point mutations in the groove. The binding model predicts that the collagen triple-helix is the major motif recognized by Cna, and that several residues in Cna interact with the collagen macromolecule. Interestingly, the resolved structures of collagen-binding regions of human integrins $\alpha_2\beta_1$ and $\alpha_1 b_1$ possess a groove where the collagen triple helix may fit (Emsley *et al.*, 1997; Rich *et al.*, 1999). This collagen-accommodating groove may be a common feature of collagen-binding proteins.

SAI/II protein of *Streptococcus mutans* consists of several domains of which the atomic structure of the central variable domain, SrV+, has been solved (Troffer-Charlier *et al.*, 2002). This domain interacts with carbohybrates on monocytes leading to the release of proinflammatory cytokines (Chatenay-Rivauday *et al.*, 1998; 2000). SrV+ has a distorted β-sandwich fold, and the crevice between two lobes in the SrV+ is suggested to be the receptor-binding site.

3.3. C-type lectin fold in intimin and invasin

Intimin of enteropathogenic *E.coli* and invasin of *Yersinia pseudotuberculosis* are virulence determinants and needed for the formation of attaching and effacing lesions in intestinal cells and for translocation into Peyer´s patches. The structure of intimin (Kelly *et al.*, 1999; Batchelor *et al.*, 2000; Luo *et al.*, 2000) and invasin (Hamburger *et al.*, 1999) share similar immunoglobulin-like (Ig) and C-type lectin-like domains in their C-terminal part, which bind to the receptor, Tir for intimin and β_1 integrins for invasin. Both adhesins are rigid rods consisting of a series of Ig-like domains that extend the C-terminal, receptor-binding domains from the bacterial surface. The C-terminal part of intimin was crystallized with the intimin-binding domain (IBD) of Tir (Luo *et al.*, 2000). The lectin-like domain of intimin and the amino-terminus of one of the α-helices in Tir IBD interact in the binding. The binding domain of intimin is reminiscent of C-type lectin domains which form a family of calcium-binding proteins responsible for cell-surface carbohydrate recognition. However, intimin lacks the calcium-binding loop of the C-type lectins and is not known to interact with carbohydrates. The integrin-binding structure of invasin also reveals an α/β topology (Hamburger *et al.*, 1999) but lacks the calcium-binding region. Although intimin and invasin share structural similarities, they differ in receptor specificity. Invasin lacks the short α-helix involved in Tir-binding and intimin is devoid of the charge configuration thought to be required for integrin adhesion (Luo *et al.*, 2000).

3.4. β-turn motif in type IV fimbrillin

The type IV fimbriae of several pathogenic bacterial species contribute to virulence. Fimbrial filament is thought to self-associate via the hydrophobic N-terminal helical residues (Forest and Tainer, 1997). The adhesive properties of type IV fimbriae are located at the tip of the fimbria (Lee *et al.*, 1994). Structural studies of the type IV fimbrillin of *Neisseria gonorrhoeae* MS11, *Pseudomonas aeruginosa* K (PAK) and K122-4 reveal a common two β-turn motif in a C-terminal disulphide loop (Parge *et al.*, 1995; Hazes *et al.*, 2000; Keizer *et al.*, 2001; Suh *et al.*, 2001). Main-chain atoms form the surface of this motif, and their conformation is important for the receptor binding. The model for type IV fimbrial fiber (Parge *et al.*, 1995; Keizer *et al.*,2001) proposes that there are five monomers of fimbrillin per the helix turn. The exposure of five monomers at the tip of the fimbria results in multivalent receptor binding.

3.5. β-barrel structure in integral membrane adhesins

The N-terminal region and surface-exposed loops of OmpA of *E.coli* contribute to adhesion to brain microvascular endothelial cells (Prasadarao *et al.*, 1996). The crystal structure of the transmembrane domain of OmpA1-171 forms an 8-stranded, antiparallel β-barrel with four extracellular loops (Pautsch and Schulz, 1998). OpcA of *Neisseria meningitidis* binds via a vitronectin bridging molecule to an integrin on endothelial cells (Virji *et al.*, 1994), and also to epithelial heparin and heparan sulfate (de Vries *et al.*, 1998). OpcA has a 10-stranded β-barrel fold with 5 external loops (Prince *et al.*, 2002). The loops of OpcA form an interesting feature: loop 2 is situated at the very top of the barrel blocking the channel and interacts with other loops to create an adhesive platform. Basic residues in the putative binding site in OpcA may accommodate heparin.

4. G FIMBRIAE OF *E.coli*

The G fimbria was first described in the *E.coli* strain IHE11165 of the serotype O2. This fimbria has affinity for terminal *N*-acetyl-D-glucosamine (GlcNAc) residues and is rare on human uropathogenic isolates (Väisänen-Rhen *et al.*, 1983; Rhen *et al.*, 1986). G fimbriae share serological and binding properties with the F17 family of fimbriae, which contains four serological variants designated F17a, F17b, F17c and F17d (Table 3); these fimbriae have been identified in animal pathogenic *E.coli* strains (Bertin *et al.*, 1996).

The expression of functional F17a fimbriae requires four genes (Lintermans *et al.*, 1991) which is less than in the more complex *pap* and *fim* operons (Hull *et al* 1981; Normark *et al.*, 1983; Lindberg *et al.*, 1984; Klemm *et al.*, 1985; Klemm and Christiansen, 1987). Mutational analysis indicated that F17-G is the adhesin (Lintermans *et al.*,1991). Amino acid sequence homology to other fimbrial proteins of *E.coli* suggest that F17-A is the major fimbrillin, F17-D a chaperone and F17-C an usher (Lintermans, 1990). The high homology of F17 subunits with Pap components suggests that F17 fimbriae may be assembled by the chaperone-usher pathway (Hung and Hultgren, 1998; Soto and Hultgren, 1999). Sequence variation in the F17-A subunits gives rise to serological variants; GafA and F17c-A have an identical sequence, whereas GafA and other F17 fimbrillins share 73-85% sequence identity (Martin *et al*., 1997).

G fimbriated bacteria agglutinate human erythrocytes treated with endo-β-galactosidase, which exposes terminal GlcNAc residues on red blood cells (Väisänen-Rhen *et al.*, 1983). *E.coli* expressing G and F17c fimbriae cause hemagglutination of bovine erythrocytes and mediate bacterial adhesion to bovine intestinal villi and to human colon carcinoma cell line Caco-2 (Bertin *et al.*, 1996) but not to human uroepithelial cells (Martin *et al.*, 1997). Hemagglutination inhibition tests show that GlcNAc binds to F17 family of fimbriae with different affinities in the order:F17c>F17b>G>>F17a>F17d (Bertin *et al.*, 1996). GlcNAc derivatives with 1β-linked methyl or benzyl group are active inhibitors of hemagglutination by F17a fimbriae (Mouricout *et al.*, 1995). Further, F17a-G binds preferentially to oligosaccharides with a GlcNAc β1-3 linkage. G fimbriated bacteria are able to bind to laminin as well as to the reconstituted basement membrane preparation Matrigel (Saarela *et al.*, 1996). Laminin is highly glycosylated and has terminal GlcNAc residues β1-3 linked to N-acetyllactosamine residues (Arumugham *et al.*, 1986; Tanzer *et al.*, 1993). The capacity to bind to laminin may potentiate G fimbriae-expressing bacteria to adhere to damaged tissue sites and to translocate to the circulation. G-fimbriated bacteria also bind plasminogen (Kukkonen *et al.*, 1998). This ability is not dependent on the GlcNAc-binding by the G fimbria since a mutated variant devoid of the lectin activity bound plasminogen. There is evidence that adhesion to laminin and plasmin formation enhance bacterial metastasis through tissue barriers (Lähteenmäki *et al.*, 1995). F17 fimbriae mediate adhesion to bovine intestinal mucin and to glycoconjugates at intestinal brush border in new-born calves (Mouricout *et al.*, 1987; 1995; Lintermans *et al.*, 1988; Sanchez *et al.*, 1993). This may enhance bacterial multiplication and colonization at the epithelia. The receptor density for F17 on epithelial cells seems to vary with the age of the calf and with the intestinal segment (Mouricout *et al.*, 1995).

Table 3. F17 fimbriae of *E.coli* (modified from Bouguénec and Bertin, 1999)

Fimbria	Adhesin	Source of isolation	Adhesins or toxins identified in the wild type strain	Reference
G	GafD	human urinary tract infection	M agglutinin	Rhen *et al.*, 1986
F17a	F17a-G	bovine diarrhoea	CNF2 toxin	Lintermans *et al.*, 1988
F17b	F17b-G	ovine bacteremia	CNF2 toxin	El Mazouari *et al.*, 1994
F17c	F17c-G	bovine septicaemia	aerobactin, CS31A adhesin	Bertin *et al.*, 1996; Martin *et al.*, 1997
F17d	F17d-G	bovine diarrhoea		Lintermans, 1990

Abbreviations: CNF2, cytotoxic necrotizing factor type 2

4.1. The G fimbrial lectin, GafD

All fimbriae belonging to the F17 family adhere to a GlcNAc-containing receptor. The in-frame deletion of two residues in GafD (Gly94-Thr95) abolished the receptor-binding ability but did not interfere with fimbriation, which is in line with the fact that GafD is the adhesin (Saarela *et al.*, 1995). GafD shares sequence identity of >95% with F17a-G and F17b-G adhesins. The *gafD* open reading frame is 1062 bp long and encodes a 321 residue mature protein. Deletion of the last C-terminal 11 amino acids from GafD abolished binding but also reduced fimbriation on the bacterial surface. This is in line with the known requirement for an intact C-terminus in the interaction with the chaperone (reviewed in Soto and Hultgren, 1999). Functional GafD has been expressed as an N-terminal fusion to MalE protein and purified using a GlcNAc-resin. Surprisingly, instead of the expected mature 32-kDa protein, a 25-kDa GafD peptide with GlcNAc-binding was detected after cleavage of the MalE fusion. Saarela and coworkers (1995) suggested that this adhesive peptide is a proteolytic cleavage product, but this has not been experimentally verified.

5. AIMS OF THE STUDY

Bacterial adhesins contribute to the virulence of several pathogenic bacteria. The understanding of the mechanisms of adhesin-receptor interactions is crucial in shedding light on pathogenic processes and on development of anti-adhesive measures. The principal goal of this study was to characterize receptor-binding epitopes of adhesins from Gram-negative bacteria. Due to the complex nature of fimbriae and instability of fimbrial adhesins, initial attempts to purify such proteins for functional and structural studies were largely unsuccessful, and alternative techniques for study of bacterial adhesive peptides were needed. Filamentous phage display is a powerful technique for identitying peptides with affinity to target molecules. In our hands, the M13 pIII-protein based display of fimbrial adhesins was unsuitable, probably due to the low affinity of individual fimbrial adhesin fragments and their large size. For this reason, a multivalent display method applicable for the study of bacterial adhesins was developed.

It became also evident that fimbrial lectins could be purified if their C-terminal proteolytic cleavage in the host bacterium was prevented, either artificially by fusion of a protease-resistant sequence at the C-terminus (Haslam *et al.*, 1994; Schembri *et al.*, 2000; Van Loy *et al.*, 2002a) or naturally as in the case of GafD. This allowed to obtain active, recombinant GafD to be purified for adhesion and structural studies.

6. MATERIALS AND METHODS

Bacterial strains and plasmids used in this study are listed in Table 4 and 5. The methods are described in detail in the original articles and are summarized in Table 6.

Table 4. Bacterial strains used in this study

Bacterial strain	Characteristics	Article	Reference
E.coli KS01	C600 *hsm hsr fliC::Tn*10	I	Kuwajima, 1988b
E.coli SM10λpir	*thi*1 *th*r1 *leuB6 supE44 tonA21 lacY1 recA*::RP4-2-Tc::Mu λ*pir*	I	Miller and Mekalanos, 1988
E.coli JT1	C600 *hsm hsr fliC::Tn*10 *fimA::cat*	I, II	This study
E.coli IHE11165	wild-type G-fimbriated strain	III	Väisänen *et al.*, 1982
E.coli IHE11088	wild-type cystitis strain	III, IV	Väisänen-Rhen *et al.*, 1984
E.coli BL21 λ(DE3)	F- *hsdS ompT lon*	III, IV	Studier *et al.*, 1990
E.coli KS474	*degP*	III	Strauch *et al.*, 1989
E.coli XliBlue MRF´	Δ(*mcrA*)183Δ(*mcrCB-hsdSMR-mrr*)173 *endA1supE44 thi1recA1gyrA96relA1lac* (F´ *proABlacI*^{q}Δ*M15Tn*10)	I, III, IV	Stratagene, Inc.
S.aureus DU5723	protein A-	I	Patel *et al.*, 1987

Table 5. Plasmids used in this study

Plasmid	Relevant property	Reference
pMMS1	*fim* gene cluster of *E.coli* PC31 in pBluescript II KS(+)	I
pMSS3	*fimA::cat* in *fim* gene cluster of *E.coli* PC31 subcloned into pGP704	I
pWQ707	*fliC*$_{H7}$ in pGEM-7Zf(+)	Schoenhals and Whitfield, 1993
pFliC	*fliC*$_{H7}$ in pBluescript II KS(+)	I
pFliCΔ	A 174 bp *Acc*I fragment in position 764-938 in *fliC* $_{H7}$ removed	I
pFR015	*fnbA* of *S.aureus* in pUC18	Flock *et al.*, 1987
pACYC184-Km	Km ^{r} gene from pHP45Ω-Km subcloned into *cat* of pACYC184	II
pD3/FliCΔ	DNA encoding D3 repeat (nt 2578-2694) of *fnbA* in *Acc*I site of pFliCΔ	I
pD2,D3/FliCΔ	as above but DNA encoding D2,D3 repeats (nt 2464-2694)	I
pD1,D2,D3/FliCΔ	as above but DNA encoding D1,D2,D3 repeats (nt 2350-2694)	I
pD1,D2,D3/FliCΔ-Km	as above but DNA encoding D1,D2,D3 repeats (nt 2350-2694) cloned into *tet* of pACYC184-Km	II
pYMS4	*yadA* of *Y.enterocolitica serotype* O3	Skurnik and Wolf-Watz, 1989
pYadA84-131/FliCΔ	fragment of *yadA* encoding amino acids 84-131 in *Acc*I site of pFliCΔ	I

pYadA84-168/FliCΔ	as above but DNA encoding amino acids 84-168	I
pYadA274-385/FliCΔ	as above but DNA encoding amino acids 274-385	I
pYadA131-274/FliCΔ	as above but DNA encoding amino acids 131-274	I
pYadA26-202/FliCΔ	as above but DNA encoding amino acids 26-202	I
pYadA84-385/FliCΔ	as above but DNA encoding amino acids 84-385	I, II
pRR-5	*gaf* gene cluster in pACYC184	Rhen *et al.*, 1986
pHUB113	*gafD* in pUC19	Saarela *et al.*, 1995
pKJ1	*gafD* in pET22b(+) with its own signal and ribosome-binding sequences	III
pGafD1-178	fragment of *gafD* encoding amino acids 1-178 in pET-22b(+)	III
pGafD1-252	as above but DNA encoding amino acids 1-252	III
pGafD1-224	as above but DNA encoding amino acids 1-224	III
pGafD1-189	as above but DNA encoding amino acids 1-189	III
pGafD1-157	as above but DNA encoding amino acids 1-157	III

Table 6. Methods used in this study

Method	described and used in
Genetic methods	
Allelic replacement	I
DNA sequencing	I, III, IV
Isolation of chromosomal DNA	I
Molecular cloning techniques	I - IV
Southern hybridization	I
Protein work	
Affinity chromatography	III, IV
Autoradiography	III
Concentration of GafD1-178	III, IV
Crystallization of GafD1-178	IV
Determination of protein concentration	I, III, IV
Expression of chimeric flagella	I, II
Expression of GafD constructs	III, IV
Extraction of periplasm and spheroplasts	III, IV
Gel filtration	III, IV
Mass spectometry of GafD constructs	III, IV
Production of SeMet- GafD1-178	IV
Protein sequencing of GafD1-132 and GafD1-178	III
Pulse-chase experiment	III
Solubility determination of GafD1-178	III
Structure determination of GafD1-178	IV
Electron microscopy	I, II
Immunological methods	
Production of antibodies	I, III
Western blotting	I, II, III
Binding assays	
Binding to GlcNAc-agarose and amylose-agarose beads	III,
Hemagglutination and inhibition assays	III,
Immunoelectronmicroscopy	I, II
Indirect immunofluorescence assay	I
Modified ELISA	I, II, III

7. RESULTS AND DISCUSSION

7.1. The variable domain of flagellin can accommodate large inserts (I,II)

A system for multivalent surface display based on the expression of the insert peptide along the flagellar filament was constructed. The expression of type 1 fimbriae was abolished by allelic replacement of *fimA* in *E.coli* C600 *hsm hsr fliC::*Tn10, also called as KS01 (Kuwajima, 1988b), that carries a silenced *fliC* but has the other genes needed for the synthesis and polymerization of functional flagellar filaments. The correct pheno- and genotype of transconjugants was assessed by (i) agglutination with yeast cells and with an antiserum against type 1 fimbriae, (ii) by electron microscopy, (iii) by Southern hybridization of chromosomal DNA (Sambrook *et al.*, 1989) with *fimA* and *cat* as probes (not shown). A transconjugant exhibiting no fimbriae and having the correct genotype was chosen and designated *E.coli* JT1.

The flagellar expression vector was constructed by subcloning the 1755 base pair (bp) coding region of *fliC* $_{H7}$ from pWQ707 (Schoenhals and Whitfield, 1993) as a *Xho*I-*Bam*HI fragment into the vector pBluescript II KS (+). To create a cloning site within the region of *fliC* that encodes the variable domain, the 174 bp *Acc*I fragment in position 764-938 of *fliC* $_{H7}$ was removed and the plasmid was religated to obtain pFliCΔ. To test the applicability of the expression vector, DNA fragments encoding D3 (39 aa), D2D3 (117 aa) or D1D2D3 (115 aa) repeats of *S.aureus* FnBPA were amplified by PCR using plasmid pFR015 (Flock *et al.*, 1987) as template and inserted in the *Acc*I site of pFliCΔ, retaining the reading frames of both *fliC* and the inserts. Further, DNA fragments encoding of 48, 85, 112, 144, 177 or 302 amino acids long fragments of the YadA adhesin of *Y.enterocolitica* serotype O3 (Figure 6A of paper I) were amplified by PCR using plasmid pYMS4 as template (Skurnik and Wolf-Watz, 1989) and inserted in the *Acc*I site of pFliCΔ. The resulting plasmids were transformed into *E.coli* JT1, and the complementation *in trans* of *fliC::Tn*10 resulted in flagella with normal morphology as assessed by electron microscopy (not shown). The obtained recombinant *E.coli* strains were motile. Chimeric flagella were purified and they reacted strongly with polyclonal anti-H7 flagella antibodies in Western blotting (see Figure 1/I for D repeats, and Figure 1/II for YadA84-385). The apparent size of the chimeric flagellins corresponded to those predicted from the nucleotide sequences. The polypeptides of smaller apparent size that were present in the preparations and reacted with the antibodies (Figures 1/I and1/II) most likely were flagellar minor proteins including the hook protein, FlgE (Figure 3/I). For consistency, the nomenclature for chimeric flagella, Insert/FliCΔ, will be used throughout this thesis.

It has been shown that large fragments from the variable region of *E.coli* flagellin can be deleted (Kuwajima, 1988b) and that short heterologous peptides up to 30 amino acids in length can be expressed at this site without affecting the polymerization or function of flagella (reviewed in Westerlund-Wikström, 2000). Flagella display has been mainly applied in construction of recombinant vaccines. Lu *et al.*, (1995) constructed a random peptide library in *E.coli* FliC, where the expression of dodecapeptides in a thioredoxin loop inserted into the varible region of FliC resulted in display of 138-residue-long heterologous peptides.We show that FliC can accomodate large inserts up to 302 amino acids without significantly affecting flagellar morphology or motility. The fact that 187 amino acids can be removed from FliC (Kuwajima, 1988b), suggests that even larger inserts can be displayed at such a construct if necessary.

7.2. Adhesive properties of chimeric flagella

7.2.1. Functional expression of D repeats of FnBPA as FliC fusions (I)

The functionality of the adhesin fragments expressed in FliC were tested. Synthetic and recombinant D repeats of *S.aureus* bind fibronectin (Fn) (Signäs *et al.*, 1989; Joh *et al.*, 1994; Huff *et al.*, 1994), which suggests that they form independent domains. The binding of purified chimeric flagella to fibronectin was assessed by enzyme-linked immunosorbent assay (ELISA), immunoelectron microscopy (IEM) and a histological staining of human cells with the flagella and an anti-fibronectin monoclonal antibody. For ELISA and histological assays, the FliC content in each flagellar preparation was assessed by SDS-PAGE and image analysis. As the ELISA assay was based on an immunological detection with anti-fibronectin and anti-H7 antibodies, the reactivity of the chimeric flagella with these antibodies was determined. The anti-H7 antibodies reacted similarly with the chimeric flagella and the deletion derivative FliCΔ, and anti-fibronectin conjugated to alkaline phosphatase did not react with the flagellar constructs (not shown).

The binding of fibronectin by chimeric flagella was dose-dependent, saturable (Figure 2/I) and equally strong with plasma and cellular fibronectin (not shown). The binding was most efficient with the flagella carrying three fibronecting-binding inserts (D1D2D3/FliCΔ) and least efficient with the flagella expressing the D3 repeat only. Flagella lacking inserts did not bind fibronectin in any of the binding assays. These assays were also performed *vice versa,* i.e. by immobilizing plasma fibronectin and testing the binding of flagella in solution; and essentially the same results were obtained (Figure 3/II). Binding to fibronectin was also visualized by IEM (Figure 3/I). The D1D2D3/FliCΔ had a thicker coating by fibronectin, anti-Fn antibody and protein A conjugate than D3/ FliCΔ (Figures 3C/I, 3B/I). The amino-terminal domain of fibronectin is the target for the D repeats (Scottile *et al.*, 1991), and the deposition of this fragment on D1D2D3/ FliCΔ was visible in electron microscope after a negative staining without antibodies (Figure 3E/I). Fibronectin bound specifically to flagellar filament polymerized of chimeric FliC and not to the flagellar hooks encoded by the *flgE,* as shown in Figure 3E/I.

The direct binding of the D repeats of FnBPA to human cells or cellular fibronectin had not been demonstrated before. Therefore chimeric flagella were tested for the ability to bind to frozen sections of human kidney in an indirect immunofluorescence assay (Korhonen *et al.*, 1986). A colocalization of the binding site of the anti-fibronectin antibody (Figure 4A) and D1D2D3/FliCΔ (Figure 4B, and 4C for FliCΔ) to glomerular mesangial areas was observed. We also assessed the binding of chimeric flagella to human embryonic skin fibroblasts (figure 4E and F), which express fibronectin well (Hedman *et al.*, 1982) and to malignant human endothelial cells expressing fibronectin poorly (Kreis and Vale, 1993) (not shown). Again, a colocalization was seen with D1D2D3/FliCΔ and anti-fibronectin antibody. Also, a quantitative difference in fibronectin binding to the two cell types was seen. The results from ELISA and the histological staining showed that flagella carrying the D repeats recognize soluble as well as cellular fibronectin.

The high affinity of D1D2D3/FliCΔ to fibronectin may result from a simultaneous binding of three D repeats to adjacent targets in the fibronectin molecule. Also, multiple adhesive motifs may have evolved in *S.aureus* to increase the affinity of FnBPA to fibronectin suggesting that

fibronectin-binding may serve as important colonization function to *S.aureus*. D repeats bind to fibronectin via their C-terminal regions (Huff *et al.*, 1994; McGavin *et al.*, 1991). Interestingly, D repeats change from a disorded to a more ordered conformation upon binding to fibronectin (House-Pompeo *et al.*, 1996; Penkett *et al.*, 2000), whether a similar conformational change is induced in the flagellar chimeras remains to be elucidated.

Flagellar filaments are good immunogens, and we raised polyclonal antibodies against D1D2D3/FliCΔ and FliCΔ and tested their anti-adhesive properties. Purified anti-D1D2D3/FliCΔ and anti-FliCΔ immunoglobulin G (IgG) antibodies were let to react with immobilized *S.aureus* DU5723 cells, which are protein A-deficient. Antibodies against D1D2D3/FliCΔ bound to the bacterial cells (Figure 5A/I) whereas no binding was detected with anti-FliCΔ (Figure 5B/I). The anti-D1D2D3/FliCΔ IgG inhibited the adhesion of *S.aureus* DU5723 to fibronectin (Figure 5C/I lane 3). The observed inhibition was only partial, probably due to the presence of multiple fibronectin-binding sites in FnBPB and FnBPA of *S.aureus* (Jönsson *et al.*, 1991; Massey *et al.*, 2001).

The anti-adhesive antibodies against FnBPs do not completely inhibit the *in vitro* adhesion of *S.aureus* to fibronectin (Ciborowski *et al.*, 1992), this could be due to several factors. Synthetic D repeat peptides used as immunogens may not be in the correct conformation to give rise to antibodies that would well block the binding (House-Pompeo *et al.*, 1996). Plasma from patients with *S.aureus* infections contain antibodies that recognize the C-terminal 20 amino acids of the D3 repeat, (McGavin *et al.*, 1991). However, the antibodies recognized the adhesin only in a complex with fibronectin and did not inhibit the fibronectin binding. Anti-adhesive antibodies that blocked the adhesion to fibronectin were produced using a synthetic peptide, which did not bind fibronectin but contained residues within the binding site of D1 or D3 (Huesca *et al.*, 2000). Such anti-adhesive antibodies are of considerable interest since they could provide means to inhibit the infection.

7.2.2. Chimeric flagella displaying a YadA fragment of 302 residues binds to collagen (I)

Flagella display was applied to identify the collagen-binding region in the YadA adhesin of *Yersinia*. The YadA peptides expressed as fusions to FliC are schematically presented in Figure 6A/I, and the binding of the YadA/FliC fusions to type IV or type I collagen were studied by a modified ELISA assay (Figure 6B/I). YadA 84-385/FliCΔ was the only construct reacting with collagens (Figure 6B lane f), the binding by the other constructs was close to the level seen with FliCΔ (Figure 6B lane g). Expression of the YadA regions reported to be involved in collagen binding (83-104 and NSVAIG-S repeat motifs in YadAO3; 80-101, 149-165 in YadAO8) (Tamm *et al.*, 1993; Roggenkamp *et al.*, 1995; El Tahir *et al.*, 2000) (see figure 6A), did not confer binding when fused separately or in combination to FliCΔ. These results indicate that the collagen-binding region in YadA is long and probably non-linear. Also other collagen-binding adhesins i.e. Cna of *S. aureus* (Symersky *et al.*, 1997), Ace of *Enterococcus faecalis* (Rich *et al.*, 1999a; Nallapareddy *et al.*, 2000) and CbsA of *Lactobacillus crispatus* (Sillanpää *et al.*, 2000) are suggested to form a conformational receptor-recognition region which can accomodate collagen. Alternatively, the correct conformation of the binding epitope in YadA may be linear and strongly influenced by other regions of the molecule. The former explanation is more likely and supported by reports that have identified several regions in the N-terminus of YadA affecting

the collagen-binding (Tamm *et al.*, 1993; Roggenkamp *et al.*, 1995; El Tahir *et al.*, 2000).

The functional expression of adhesive D repeats and YadA peptides in chimeric flagella as well as the recent identification of a fibronectin- and cell-binding domain in an S-layer protein of *Lactobacillus brevis* (Hynönen *et al.*, 2002) indicate that the flagella display can be successfully used in ligand-receptor studies. Flagella support multivalent display i.e. the D repeats and YadA fragments were expressed along the filament; in theory the filament contains 20 000 copies of FliC (Macnab, 1996). Multivalency increases the avidity of low-affinity molecules, which is an advantage in display of adhesive epitopes. Expression along a fimbrial filament also promotes multivalency but the epitopes displayed on fimbriae have been fairly short (reviewed in Klemm and Schembri, 2000). The affinity of binding by the chimeric flagella was not determined in this study, but multivalency and the tolerance of large insert size makes flagella a powerful display carrier.

A disadvantage of flagella display is that the flagellar secretion apparatus bypasses the periplasmic space of *E.coli* where cysteines are oxidized, thus disulphide bonds are not formed in FliC and hybrid FliC molecules. Our experience in expressing peptides of fimbrial adhesins showed that chimeric flagella are formed but not functional in binding assays. This probably resulted from the lack of the functionally important disulphide bond (Carnoy and Moseley, 1997). For example, the successful expression of DraE and peptides thereof in FliC was verified by Western blotting with anti-Dr antibodies but the hybrid FliC molecules failed to bind to type IV collagen (unpublished). This is in line with the finding by Carnoy and Moseley (1997), that mutagenesis of the two cysteines in DraE abolishes binding to type IV collagen. Attempts to oxidize the cysteine residues in DraE/FliCΔ with disulphide isomerase, DsbA, failed (unpublished). On the other hand, Lu *et al.* (1995) reported expression of random peptides in a thioredoxin active site loop fused in FliC. This FLITRX system is believed to display 12mer inserts in a constrained disulphide loop of the thioredoxin peptide, but the presence of disulphide bonds in the chimeric flagella was not demonstrated.

7.2.3. Construction of a bifunctional flagella (II)

In various applications, it would be useful to simultaneously express more than a single heterologous insert, and we approached this by using the D1D2D3 repeats and the YadA84-385 as model peptides. DNA fragment containing the D1D2D3 repeats in the *fliC*Δ was subcloned into the *tet* gene of plasmid pACYC184-Km to obtain an expression plasmid compatible with pBluescript. The bihybrid complementation strain *E.coli* JT1 (pD1D2D3/FliCΔ-Km)(pYadA84-385/FliCΔ) was designated BFS1. The complementation of the silenced *fliC* in JT1 with each plasmid individually or simultaneously resulted in expression of flagella with normal morphology as seen by electron microscopy (not shown). Western blotting with anti-H7 antibodies of flagella from *E.coli* BFS1 showed that the chimeric flagellins were expressed equally well (Figure 1/II). Two major polypeptides, corresponding in size to D1D2D3/FliCΔ (69 kDa in apparent size) and YadA84-385/FliCΔ (87 kDa), were detected. The results showed that both flagellins were expressed at the same time and with similar efficiency in *E.coli* BFS1(Figure 1 lane 1).

We used IEM to analyze whether the two inserts were expressed along the same flagellar filament. YadA fragments were visualized by monoclonal anti-YadA antibody and secondary antibodies conjugated to colloidal gold particles of 5 nm in diameter, wheras D repeats were visualized with fibronectin, anti-fibronectin and a protein A conjugate with gold particles of 10 nm in diameter. Bihybrid flagellar filaments (Figure 2A to C) bound anti-YadA antibodies as well as soluble fibronectin, and double staining of the flagella revealed both small and large gold particles along single flagellar filaments (Figure 2A). The D1D2D3/FliCΔ hybrid flagella (Figure 2D to F) bound fibronectin but not anti-YadA antibodies, and YadA84-385/FliCΔ flagella (Figure 2G to I) reacted with the anti-YadA antibodies. Binding of fibronectin to D repeat-containing flagella was seen as massive coat on the flagella. Control flagella lacking inserts (Figure 2J to L) did not interact with soluble fibronectin or anti-YadA antibodies. No immunostaining was observed in control samples lacking one of the reagents in the mixture (not shown).

Microtiter plate was coated with fibronectin, type IV collagen and fetuin (control) and proteins were let to react with flagella in order to assess the functionality of the inserts in the BFS1 flagella. Bihybrid flagella bound to immobilized fibronectin and type IV collagen but not to fetuin and the binding was dose-dependent and saturable (Figure 3). YadA84-385/FliCΔ also bound fibronectin weakly (Figure 3A) which is in line with the finding by Tamm *et al.* (1993) that YadA binds strongly to laminin and collagens and only weakly to immobilized fibronectin. YadA does not bind to soluble fibronectin (Tertti *et al.*, 1992) which explains the lack of activity of YadA84-385/FliCΔ in the immunoelectron analyses shown in Figure 2/II.

These results showed that hybrid flagellins were expressed and polymerized in the same filament with approximately equal frequency. The inserted peptides retained their adhesive properties and did not sterically interfere with each other´s function. It was also shown that chimeric flagella can be used in whole cell formats or as soluble, purified hybrids, which enables their use in adhesion studies as soluble or immobilized ligands. Flagellin seems to be quite permissive for inserts of differing size and chemical properties, as the D repeats and YadA vary in their pI and charge and come from evolutionary distant organisms. Bifunctionality in fimbriae has been composed of the natural functionality of the fimbriae and of the display of a novel function such as metalloadsorption (Kjaergaard *et al.*, 2000; Schembri and Klemm, 1998) or antigenicity towards viral epitopes (Rani *et al.*, 1999). Short inserts up to 56 residues have been successfully displayed on bifunctional fimbriae (Pallesen *et al.*, 1995), which is a limitation of this display system.

7.3. A 178 residue GlcNAc-binding domain of GafD (III)

Saarela *et al.*, (1995) expressed GafD-MalE fusion protein and after cleavage of the fusion detected only a 25-kDa GafD form that bound to GlcNAc. The protein yields were modest, and it was difficult to separate GafD from the fusion partner. We expressed *gafD* in a non-fusion form using a pET-expression vector, which provides expression under the tight control of T7 promoter. *gafD* was subcloned from pHUB113 into pET-22b(+) to obtain plasmid pKJ1, where it was expressed with its own ribosome-binding and leader sequences with stop codons upstream and downstream of the reading frame. The peptide thus lacks the vector-encoded PelB leader as well as the His-tag sequence. We expressed GafD to the periplasm of *E.coli* BL21λ(DE3) either

as metabolically labelled or as a nonlabelled protein, depending on the use of the peptide. GafD was released from the periplasm by osmotic shock and purified by affinity chromatography on GlcNAc-agarose, subsequently GlcNAc was removed by gel-filtration.

The binding of radiolabelled GafD to GlcNAc-agarose is shown in Figure 1A/III. Three radioactive peptides were detected in the periplasm of BL21λ(DE3)(pKJ1) (lane 1). The minor peptide with an apparent molecular mass of 32 kDa bound to GlcNAc-agarose (lane 3) and was eluted with GlcNAc-PBS, which indicated that it was GafD (calculated size 33 889 Da). The 20 kDa periplasmic peptide bound also to GlcNAc-agarose (lane 3) and was eluted in GlcNAc-PBS (Figure 1B, lane 1). The 30 kDa peptide did not bind to GlcNAc-agarose (Figure 1A, lane 2) and was the vector-encoded β-lactamase (Figure 1A, lane3). Anti-G fimbriae antibodies recognized the purified 20-kDa peptide in Western blotting (not shown) and the amino-terminal peptide sequence AVSFIGS perfectly matched the amino-terminal sequence of the mature GafD (Saarela *et al.*,1995). These findings indicated that the peptide was C-terminally truncated, and was designated ΔGafD. Mass spectrometry of the ΔGafD peptide yielded a mass of 19 092+/- 20 Da, which is close to the apparent size of 20kDa detected by SDS-PAGE. The determined molecular mass indicates that the C-terminal residue in ΔGafD is Thr-178 (calculated molecular mass, 19 074 Da). The solubility test indicated that ^{14}C-ΔGafD was soluble. The addition of commercially available mixture of serine and cysteine protease inhibitors to the induction broth did not prevent degradation (not shown).

To determine whether ΔGafD is incorporated into the G fimbrial filament and whether it was present in other host backgrounds as well, Western blotting of peptides in G fimbrial preparations and in periplasmic extracts were performed with anti-G-fimbrial and anti-GafD antibodies. Full-length GafD was present in the fimbrial preparations (Figure 1C/III), whereas ΔGafD was not detected. Plasmid pRR-5 containing the *gaf* gene cluster was introduced into *E.coli* strains deficient in cytoplasmic protease Lon and outer membrane protease OmpT or periplasmic protease DegP. GafD appeared as a minor 32-kDa peptide and ΔGafD as the major form in the periplasms of these host strains (lanes 1 and 2 in Figure 2B). The same was observed in the periplasmic extracts of *E.coli* IHE11088 (pRR-5), which is a clinical isolate, and *E.coli* IHE11165, from which the *gaf* gene cluster was originally isolated (lanes 3 and 5). These *E.coli* strains were also positive in hemagglutination assays for a functional GafD (not shown). To determine whether ΔGafD is a result of proteolytic activity rather than a primary translation product, pulse-labelled cells of BL21λ(DE3)(pKJ1) were analyzed by autoradiography. Samples were taken immediately after the addition of ^{14}C-labelled amino acids and at short intervals up to 1h 30 min after addition of the label. Intact GafD and trace amounts of 20- and 30-kDa peptides corresponding to ΔGafD and vector-encoded β-lactamase were detected immediately after adding the label (lane 1 in Figure 2A). The amount of ΔGafD increased over time (not shown) and was clearly visible 1h 30 min after label addition (lane 2 in Figure 2A).

The finding that also *E.coli* IHE11165 supported formation of ΔGafD indicates that the ΔGafD form is a natural product of the *gaf* gene cluster and not a result of the overproduction technology. The truncate was not detected in fimbrial filaments, which indicates that ΔGafD is not competent for assembly. This finding is in agreement with the results by Saarela *et al.*, (1995) showing that an 11 amino acid deletion at the C-terminus of GafD reduced fimbriation in *E.coli* and abolished the receptor-binding activity. ΔGafD appears not to be a translation product but results from proteolytic degradation. Degradation of GafD also occured in Lon and OmpT or

DegP-deficient *E.coli* and the proteases responsible for the C-terminal processing remain unidentified. No significant homology of the GafD region around residue Thr-178 to the known target sequences of *E.coli* proteases (Gottesman, 1999) was observed.

It has been reported that fimbrial proteins are degraded in the absence of their chaperone (Tewari *et al.*, 1993; Hultgren *et al.*, 1989; Slonim *et al.*, 1992; Barnhart *et al.*, 2000) and also when their cognate chaperone is coexpressed (Jones *et al.*, 1993; Barnhart *et al.*, 2000). Truncated forms of PapG and FimH were C-terminally processed but retained their receptor-binding ability (Hultgren *et al.*, 1989; Jones *et al.*, 1993), in parallel with ΔGafD. So far, there have been no reports on the presence of a truncated fimbrial adhesin in wild-type strains. The finding obtained here raises the interesting possibility that fimbrial lectins have also nonadhesive functions in the bacterial cell, their nature, however, remains to be elucidated.

7.4. GafD1-178 forms a stable domain (III)

To determine the minimum size of the receptor-binding domain of GafD, C-terminally truncated mature peptides GafD1-252, GafD1-224, GafD1-189, GafD1-178 and GafD1-157 were constructed by PCR-cloning into pET-22b(+) using the plasmid pHUB113 as a template (Figure 3A/III). Autoradiographic analysis of their expression in *E.coli* BL21λ(DE3) cells, spheroplasts and periplasm revealed that the complete GafD (^{14}C-GafD1-321) migrated in whole cells and spheroplasts mainly as a 32-kDa peptide (Figure 3B lanes 1 and 2) but appeared in the periplasm as a 20-kDa peptide as expected from the previous experiments (lane 3). This demonstrates that GafD is not processed until it reaches the periplasmic space and indicates that the C-terminus of GafD is resistant to cytoplasmic proteases. The shortest form, GafD1-157, appeared in the cells and spheroplasts as a 15-kDa peptide but was not observed in the periplasm of pulse-labelled cells (lanes 4 to 6). The GafD1-252, GafD1-224, GafD1-189 and GafD1-178 peptides were detected in cells, spheroplasts and periplasm with an apparent molecular weight of 20 kDa , which suggested that they were proteolytically processed to a stable structure already in the cytoplasm (GafD1-252 and GafD1-178 are shown in lanes 7 to 12 in Figure 3B/III). GafD1-178 was subjected to mass spectrometry, and the result gave a mass of 19113 Da, which is reasonably close to its calculated molecular mass (19074 Da) and to the observed mass of ΔGafD (19092 Da). This indicated that GafD1-178 forms a stable structure. The 32-, 20- and 15-kDa peptides in whole cell samples reacted in Western blotting with anti-GafD antibodies (not shown), which indicated that these peptides originated from GafD. The truncted GafD molecules bound GlcNAc-agarose, except for the GafD1-157, indicating that the 178 amino-terminal residues are responsible for the receptor recognition.

The fact that GafD was not processed in the cytoplasm but appeared in the periplasm as C-terminally truncated peptide suggests that the C-terminus protects GafD from cytoplasmic degradation. Reports indicating the protective role for the C-terminus of fimbrial proteins (Haslam *et al.*, 1994; Schembri *et al.*, 2000; Van Loy *et al.*, 2002) are in line with our results.

7.5. Binding characteristics of ΔGafD (III)

The carbohydrate specificity of ΔGafD was assessed by the inhibition studies using GlcNAc-agarose as the binding target. The binding of ΔGafD to GlcNAc was specific, since it was inhibited by GlcNAc but not with D-glucose, 2-deoxyglucose, *N*-acetyl-D-galactosamine (GalNAc), D-glucosamine or D-mannose (Table 1/III). *N*-acetyl-D-mannosamine (ManNAc) was not as potent inhibitor as GlcNAc, whereas GlcNAc-oligosaccharides, which have GlcNAc chains in β1-4-linkage, inhibited the binding only marginally better than the GlcNAc monosaccharide. A similar inhibition pattern was obtained in hemagglutination assays with G-fimbriated *E.coli* (Table1/III). The basement membrane glycoprotein laminin is a tissue target for the G fimbria (Saarela *et al.*, 1996). Adherence of ΔGafD to immobilized laminin was tested in an ELISA assay(Figure 4/III). A dose-dependent binding to laminin was detected (Figure 4A) and the binding to laminin was inhibited by GlcNAc but not by GalNAc (Figure 4B). This finding and the fact that the two inhibition assays gave closely similar inhibition patterns, indicate that ΔGafD expresses the full binding specificity of the G fimbriae.

7.6. GafD 1-178 cocrystallized with GlcNAc yields an atomic structure (IV)

The fact that GafD1-178 forms a stable and soluble receptor-binding domain implied that this peptide is suitable for structural studies. GafD1-178 was expressed in *E.coli* BL21λ(DE3)(pGafD1-178) in batch cultures (5 to 15 l) and periplasmic extract was purified and concentrated to 10-30mg/ml. The yield of the purified GafD1-178 was low, due to low expression levels (900μg/l) and loss of protein during purification and concentration. Concentrated GafD1-178 was crystallized using sitting drops in the presence of GlcNAc remaining after the purification. Crystals grew slowly as thin plates and were irreproducible. Microseeding yielded plates of 400 μm x 250 μm x 50μm after 10 days at 4°C. Crystals gave native diffraction data beyond 1.7 Å on a conventional rotating anode source. GafD1-178 crystallized in the monoclinic space group of $P2_1$ with unit cell dimensions of 42.6 Å, 70.3 Å, 56.8Å, 90.0°, 104.9°, 90.0°. A calculated solvent content of 42.9% and a Matthews coefficient of 2.2 $Å^3$ Da^{-1} implied that there are two molecules in the asymmetric unit that are not related by a two-fold rotation and do not form a closed point group. Selenomethionine (SeMet) was used as a replacement for methionine to provide a heavy atom derivative for phase determination. SeMet was incorporated into the GafD1-178 by metabolic inhibition (Van Duyne *et al.*, 1993) and incorporation of SeMet was confirmed by mass spectrometry. One SeMet-crystal diffracted to 2.5 Å resolution and was used for multiwavelength anomalous dispersion (MAD) phasing. The data yielded electron maps with a clearly defined molecule/solvent boundary and recognizable bacbone features. The refined 2.5 Å model was used as a search probe for molecular replacement against the 1.7 Å dataset. The 1.7 Å model was refined in a standard fashion (Table 1/IV).

7.7. Structure of the receptor-binding domain of GafD 1-178 (IV)

GafD1-178 consists of 16 β-strands and one 3/10-helix (Figure 1/IV), which is a distorted α-helix with three residues per turn and with 10 atoms in the hydrogen-bond loop. GafD1-178 is a prolate ellipsoid with axial dimensions of 63.7, 31.6 and 26.1 Å. The overall structure of GafD1-178 is a β-barrel with a jelly roll-like fold, excluding β-strands 4 and 5.

The electron density maps showed extra density consistent with two bound GlcNAc molecules while crystals grown in the absence of added GlcNAc showed only one bound monosaccharide. The GlcNAc remaining after affinity purification is apparently tightly bound and not easily removed by gel filtration, and so defines the GlcNAc recognition site of GafD1-178. The receptor-binding site of GafD1-178 resides on the side of the molecule in an extended cleft formed by the C-terminal end of strand 6, the 3/10 helix, and strands 7,8 and 9 (Figure 1A/IV). GafD1-178 interacts with GlcNAc mainly through hydrogen bonds: six side chain (Asn44, Asp88, Thr89, Arg93, Thr117,) and two main chain (Gly119, Ala43) hydrogen bonds with the receptor (Figure 3/IV). Ser116 makes a water-mediated hydrogen bond with GlcNAc. Trp109 creates a hydrophobic contact with GlcNAc, the indole ring is parallel to the plane of the sugar ring. Specificity for GlcNAc resides, apparently, in the arrangement around Thr117. Thr117 positions the acetamide group by interacting with the nitrogen and the carbonyl group (Figure 4). Asn44 hydrogen bonds with the acetamide carbonyl group, while Ala43 interacts with C3 oxygen of GlcNAc. Only the N-acetyl group in GlcNAc can form this complete set of interactions. A disulphide bridge located in strands 3 and 8 provides extra rigidity to the binding site and especially to the region that interacts with the acetamide group. The disulphide bond connects binding site residues Trp109 (strand 8) and Ser116-Thr117 (strand 9) to Ala43-Asn44 (before strand 3) and thus constrains the region required for GlcNAc specificity (Figure 4/IV). The deletion mutant Gly94:Thr95 of GafD has been shown to be deficient for binding (Saarela *et al.*, 1995). These residues occur just before the 3/10-helix and the loop carrying Asp88. Shortening this region would probably alter the loop and affect the ability of Asp88 to interact with GlcNAc. We noticed that the substitution D88L in GafD1-178 reduced GlcNAc-binding by ca. 80% (not shown), and supposedly, the bidentate hydrogen bond is important in the binding.

Glucose, GalNAc and ManNAc do not inhibit the binding of ΔGafD to GlcNAc (Tanskanen *et al*., 2001), which supports the GafD1-178 structural data i.e. only the acetamide group of GlcNAc is able to form the correct interactions with theGafD1-178 binding site. The binding pocket is large enough to accommodate another monosaccharide as GlcNAc alone may not be the physiological receptor but may be linked to yet unidentified structures. Arg93 is located in close proximity to bound GlcNAc, and this raises the possibility that it interacts with charged groups in GlcNAc-containing structures, such as a $-SO_3$ moiety in a heparan sulfate.

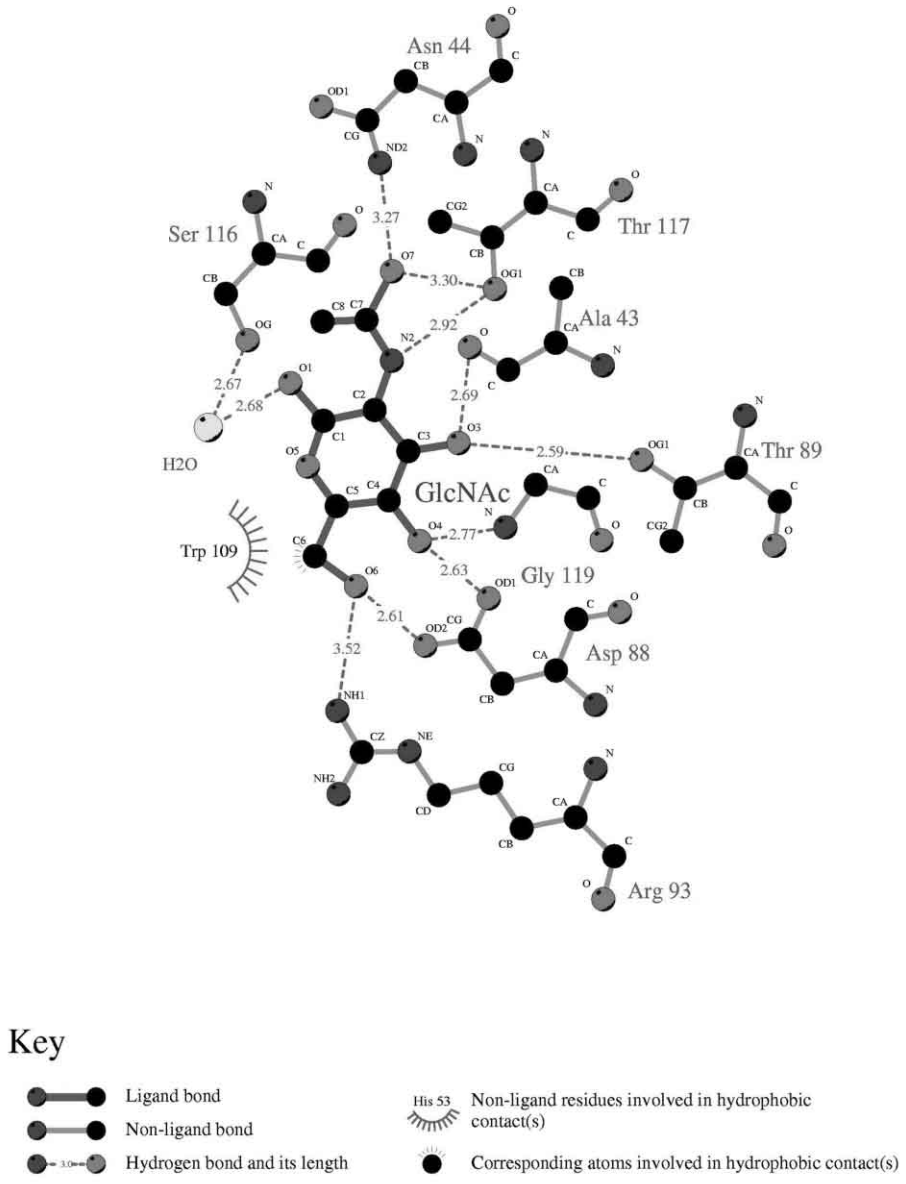

Figure 4. A schematic presentation of the GlcNAc binding-site.

7.8. GafD 1-178 structure is related to FimH (IV)

GafD1-178 ,FimH and PapG adhesive domains fold similarly although GafD1-178 has only ca. 21% amino acid identity with these lectins (Figure 2/IV). These proteins are all elongated molecules 50-70 Å long and 26-33 Å wide. The receptor-binding sites are all located in the upper half of the domains and the topology around the GafD1-178 binding-site is preserved in all three lectin domains. The structures of GafD1-178 and FimH seem to be more closely related to each other than the larger PapG domain as indicated by the structural analysis (not shown). GafD1-178 topology can be derived from FimH by two major changes (Figure5/IV). First, by deleting the strand 2 of FimH. Secondly, by replacing the short α-helix of FimH with a β-hairpin made by GafD strands 4 and 5. The 3/10-helix of GafD1-178 occurs between strands 6 and 7. This region includes Asp88 and so the change between the two lectins may be due to the development of the GlcNAc-binding site in GafD.

A disulphide bond in GafD1-178 was elucidated, and the disulphide bond most probably stabilizes the structure strand 8-turn-strand 9 of the binding site. The cysteines found in the adhesive domain of FimH were not reported to form a bridge (Choudhury *et al.*, 1999; Hung *et al.*, 2002). The report of Sung *et al.*, (2001) demonstrated the presence of a disulphide bond between Cys44 and Cys118, which could stabilize two strands that are part of the binding site of PapG. The putative disulphide bond of FimH and the ones of GafD1-178 and PapG are situated in the adhesive part of these proteins but there is no spatial correlation between the bonds. The significance of a disulphide bond in receptor-binding was evident in DraE, which lost its functionality upon mutation of the cysteine residue or disruption of the disulphide bond (Moseley and Carnoy, 1997).

Although each adhesin is made from topologically similar jelly roll β-barrel fold, they differ in receptor-binding site location as well as disulphide bond patterns. GafD and PapG bind their receptors on one side of the molecule whereas mannose binds to the tip of the FimH. The shape of the carbohydrate-binding pocket is dissimilar between the three lectins; GafD1-178 has an extended cleft with a surface volume of 329 $Å^3$, which is smaller than that of the deep pocket of FimH with a large interaction surface with D-mannose (459 $Å^3$) but larger than the shallow globoside (GbO4) site of PapG, 284 $Å^3$. Thus the carbohydrate-binding region of GafD1-178 is closer to that of the PapG. It has been speculated that shape, size and location of the receptor-binding pocket influence the binding specificity of the adhesin, a shallow and extended pocket allows a more flexible specificity whereas a small and deep pocket provides a more narrow specificity and high affinity (Dodson *et al.*, 2001; Hung *et al.*, 2002). GafD1-178 interacts with GlcNAc mainly via hydrogen bonds. Interestingly, the indole ring of Trp109 is situated parallel to the plane of GlcNAc and may attract the carbohydrate via hydrophobic interaction closer to the binding site. A similar arrangement of Trp and sugar is seen with PapG and GbO4 (Dodson *et al.*, 2001). The hydrophobic ring around the FimH mannose-binding pocket suggests a related mechanism (Hung *et al.*, 2002).

8. CONCLUSIONS

Bacterial adhesins recognize the target molecules often by conformational epitopes and with low affinity. This leads to challenges in the available display formats. We developed a multivalent flagella display system that can adopt large inserts and in multiple copies. The model peptide, the D repeats of *Staphylocccus aureus* FnBPA, as well as the YadA peptide from *Yersinia*, were expressed in a functional form. The fact that chimeric flagella bound to human tissue sections and cells, suggests that such flagella can be developed as tools in detecting, localizing and quantitating receptor-active tissue domains in disease-susceptible individuals. Our studies also indicate that chimeric flagella could be used to raise anti-adhesive antibodies, which is a major aim in design of anti-adhesive measurers.

The multivalency of the flagella display allows a possibility to display several epitopes simultaneously along the filament, which was demonstrated in this study by the creation of bihybrid chimeric flagella. The two adhesive inserts were equally expressed and did not interfere with each other´s function. Bifunctional flagella display would be beneficial in expressing two or more antigenic epitopes in a vaccine strain, constructing targeted effector molecules carrying targeting as well as effector peptides, and in localizing specific tissue domains for diagnostics.

On-going work in our laboratory (Salonen *et al.*, submitted) has expanded the flagella technology to secrete N-terminal FliC fusion peptides through the flagellar secretion apparatus into the culture medium. Such soluble, secreted peptides are likely to have less structural constraints than the present flagella strategy. A pronounced limitation of the flagella display system is that the formation of disulphide bonds is not feasible during the biogenesis of FliC. In particular, fimbrial adhesins contain cysteines, Carnoy and Moseley (1997) have shown that the disulphide bond in the Dr adhesin is essential for binding and the disulphide bond in GafD structure may stabilize the binding region.

The structure of the G fimbrial adhesin domain GafD1-178 was determined here. This is the third *E.coli* fimbrial adhesin structure reported to date, and the structure gives information on the adhesive mechanisms as well as the evolution of fimbrial lectins. The size and shape of the resolved lectin domains are similar, i.e. elongated and rich in β-strands, which may be a requirement for translocation via the chaperone-usher pathway onto the growing fimbria on the cell surface (Hung *et al.*, 2002). The adhesive domain of GafD resembles that of FimH although their binding sites differ in location, conformation and specificity. They most probably can accomodate more than one monosaccharide, GafD has space for a disaccharide and the deep pocket of FimH for a trisaccharide. Binding of FimH to mono- and trimannose moieties correlates with increased uropathogenicity (Sokurenko *et al.*, 1995; 1997; 1998). One or more hydrophobic residues (e.g. Trp109 in GafD that is parallel to the GlcNAc plane) in the immediate vicinity of the binding site (Hung *et al.*, 2002) may attract sugar moieties and be a common feature for fimbrial lectin activity.

The few existing structures of bacterial adhesins give a limited possibility for comparison with the results obtained with fusion or display technology. The N-terminal portion of PapG was found functional as a MalE fusion (Haslam *et al.*, 1994), and the identified binding region fits well with the structural data obtained later. Knudsen and Klemm (1998) created FimH-FocH hybrid (FocH is the type-1C fimbrial adhesin subunit) and localized the FimH binding region to

the first 158 residues. PapG fragments derived from the N-terminal region and fused to OmpS bound globoside and lactosylceramide (Lång *et al.*, 2000), and the crystal structure determination revealed that the binding region is larger than the N-terminal 53 residues identified by Lång *et al.* (2000). The P fimbriae are specific for globoside, and the results by Lång *et al.* exemplify that expression of a partial binding site may modify the target specificity.

The structure of GafD1-178 did not reveal any significant clues on why this peptide is so stable. The Thr178 is not buried in the binding domain but is exposed at a site close to the putative linker region. The C-terminus of a fimbrial adhesin evidently plays a role in protection against proteolysis (Haslam *et al.*, 1994; Schembri *et al.*, 2000; Van Loy *et al*., 2002a). A mechanistic explanation for the protease resistance of GafD1-178 remained unanswered, it obviously was resistant to cytoplasmic and periplasmic degradation. Whether the two-domain structure of FimH is shared with other fimbrial adhesins and whether the naturally occurring ΔGafD provides a paradigm for a naturally occurring truncated fimbrial lectin remains to be elucidated.

ΔGafD was completely soluble and expressed the same carbohydrate specificity as the entire G-fimbrial filament. Such soluble, active fimbrial lectins offer ideal tools to identify and quantitate receptor molecules on target tissues.

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