

Functional expression of adhesive peptides as fusions to *Escherichia coli* flagellin

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An expression system for studying epitopes of adhesion proteins based on fusion of gene fragments into *fliC*_{H7} of *Escherichia coli* is described. We constructed the system by an in-frame insertion of DNA fragments encoding one, two or three of the fibronectin-binding D repeats present in the fibronectin-binding protein A (FnBPA) of *Staphylococcus aureus*, into the *fliC*_{H7} gene region encoding the variable domain of the H7 flagellin. The constructs were expressed by *in trans* complementation in the *E. coli* strain JT1 which harbours knock-out mutations for the expression of FliC as well as of the mannoside-binding fimbrial adhesin. The resulting chimeric flagella, which contained 39, 77 or 115 heterologous amino acid residues, efficiently bound soluble and immobilized human plasma and cellular fibronectin, and the binding was most efficient with the flagella containing the three D repeats of FnBPA. The chimeric flagella bound to frozen sections of human kidney and to cultured human cells. Antibodies raised against the chimeric flagella bound to Protein A-deficient *S. aureus* cells and inhibited the binding of staphylococci to immobilized fibronectin. We also expressed peptides, ranging in size between 48 and 302 amino acids, of the collagen-binding YadA adhesin of *Yersinia enterocolitica*. A fragment of 302 amino acids representing the middle region of YadA was needed for collagen binding. Chimeric flagellar filaments expressing hundreds of intimately associated adhesive epitopes offer versatile tools to analyze adhesion–receptor interactions and functional epitopes of adhesion proteins.
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Introduction

Molecular interactions provide the specificity for all forms of cell-to-cell contact and are essential for the attachment of eukaryotic cells to their surroundings, and for prokaryotes to host tissues. Specific interactions are crucial for cellular development, proper function, survival, as well as tissue tropism. Hence functional and structural analysis of adhesion proteins—both prokaryotic and eukaryotic—and their receptors represents a biologically highly important topic with true potential for biotechnological applications. For pathogenic bacteria, for example, attachment to host tissues is critical

for the development of infectious disease (Karlsson, 1989; Korhonen *et al.*, 1990). Adhesion to epithelial surfaces helps the bacteria to colonize tissues and resist the cleansing defence mechanisms of the human body. Pathogenic bacteria also frequently adhere to subepithelial extracellular matrix (Westerlund and Korhonen, 1993; Patti and Höök, 1994), which is considered important in infections initiating at damaged tissue sites (Switalski *et al.*, 1993) as well as in bacterial penetration through basement membranes (Lähteenmäki *et al.*, 1995). Finally, bacterial attachment to epithelial receptors is the first phase in invasion of the epithelium and in triggering epithelial cell responses to invading pathogens (Bliska *et al.*, 1993). Tissue receptor density for bacterial adhesins varies in different tissue compartments (Korhonen *et al.*, 1990) and is a key factor predisposing certain individuals to repeated infections (Källenius and Winberg, 1978).

The primary structure of a number of bacterial adhesins have been determined. Adhesins of Gram-positive bacteria are typically large multidomain surface proteins that exhibit multiple binding specificities (McGavin *et al.*, 1993; Patti and Höök, 1994) and may occur in several variants in a bacterial strain (Jönsson *et al.*, 1991). Fimbriae of Gram-negative bacteria are filamentous adhesive complexes that carry the adhesive minor subunit in a few copies at the tip of the filament (Hultgren *et al.*, 1991). Many of the fimbrial adhesion mechanisms are of low affinity, which has complicated the analysis of their tissue-type specificity as well as of their tissue receptors. There is a need to construct efficient expression systems for adhesion genes and their fragments that would allow the study of specific binding epitopes and their tissue interactions. Genes encoding bacterial adhesins or their fragments have been expressed as fusions to proteins or peptides functioning as affinity handles, such as the maltose-binding protein, glutathione S-transferase or polyhistidine-tail (McDevitt *et al.*, 1995; Patti *et al.*, 1995; Saarela *et al.*, 1995). Jacobsson and Frykberg (1995) recently used fusion into the gp3 protein of filamentous phage to clone adhesive epitopes from the chromosome of *Staphylococcus aureus*. In these systems, the adhesive epitope is expressed as a single copy per fusion protein, or at best, as three copies on the surface of the filamentous phage, which restricts the use of these approaches when adhesive epitopes of low affinity are under study. On the other hand, fusion of foreign epitopes to the major capsid protein (gp8) of the M13 phage allows expression in a multicopy system but is limited to expression of short oligopeptides (Illichev *et al.*, 1989; Felici *et al.*, 1991).

Our reasoning was that by expressing adhesive epitopes in thousands of intimately associated copies along the flagellar filament in the variable region of FliC, we might construct high-affinity expression systems for the analysis of adhesion–receptor interactions. The bacterial motility organelle flagellum is a long filament composed of several thousand copies of the FliC major protein as well as a few copies of the tip-associated FliD protein and the junction proteins FlgL and FlgK. The

FlgE protein forms the polymeric hook structure that anchors the flagellum into the basal body of the cell wall (DeRosier, 1992). The primary structure of eubacterial flagellins (FliC) is highly conserved at the N- and the C-termini, whereas the central region of FliC forms a solvent-exposed, variable domain (Wilson and Beveridge, 1993). Up to 187 amino acid residues at this domain can be deleted without loss of flagellar polymerization or motility (Kuwajima, 1988b), and this region is also responsible for the antigenic variability in bacterial flagella (Kuwajima, 1988a). Fusions into the central region of FliC have been constructed to express short, 15–26 amino acid-long antigenic epitopes of viral proteins (Wu *et al.*, 1989) and cholera toxin (Newton *et al.*, 1989). Upon vaccination, the resulting chimeric flagella evoked antibodies recognizing the inserted epitopes.

To develop a method for identification and analysis of adhesive epitopes, we constructed chimeric flagella using as model epitopes the well characterized fibronectin-binding D repeats of the fibronectin binding protein A (FnBPA) of *Staphylococcus aureus* (Flock *et al.*, 1987). FnBPA carries three adjacent repeats termed D1, D2 and D3, as well as a truncated repeat D4. D1, D2 and D3 are 38 or 39 amino acids each, and their sequences are highly homologous (Signäs *et al.*, 1989). We chose to use the D repeats as the test adhesive ligands in this study because evidence from inhibition and binding assays (Signäs *et al.*, 1989; Joh *et al.*, 1994; Huff *et al.*, 1994) suggests that the D repeats are functional adhesive domains. On the other hand, a direct binding of the D repeats to human cells or cellular fibronectin has not been demonstrated.

We applied the flagella display to locate the collagen binding region in the YadA adhesin of the Gram-negative bacterium *Yersinia enterocolitica*. YadA is considered the most important single virulence factor of *Y. enterocolitica*. It is a 44–47 kDa protein that forms a polymeric layer on the bacterial surface (Zaleska *et al.*, 1985) and mediates bacterial adherence to a number of tissue components such as laminin and various collagens (Tamm *et al.*, 1993; Roggenkamp *et al.*, 1995). Binding of YadA to collagens potentiates the dissemination of yersinias to secondary infection foci. YadA represents a bacterial S-layer protein in primary structure and in being hydrophobic and easily aggregated (Gripenberg-Lerche *et al.*, 1995; Kapperud *et al.*, 1987), which has remained a severe limitation in the functional analysis of these important surface proteins (Sleytr and Sára, 1997).

Materials and methods

Bacterial strains and growth conditions

Escherichia coli C600 *hsm hsr fliC::Tn10*, also called KS01 (Kuwajima, 1988a), was kindly donated by Goro Kuwajima (Shionogi Institute for Medical Science, Osaka, Japan). The Protein A-deficient *S. aureus* strain DU5723 was available from previous studies (Patel *et al.*, 1987). *E. coli* strain SM10 λ pir (Miller and Mekalanos, 1988) was used as a donor in conjugation in the construction of strain JT1. For conjugation, bacteria were grown in Luria broth for 16 h at 37°C and for production of flagella, strains were grown on Luria plates for 72 h at 28°C. For immunofluorescence and adhesion assays, staphylococci were grown on Luria plates for 18 h at 37°C. When appropriate, antibiotics were added at the concentrations of 100 (ampicillin; Ap), 25 (chloramphenicol; Cm), 50 (kanamycin; Km) and 20 µg/ml (tetracycline; Tc).

DNA techniques

Plasmid and chromosomal DNA were isolated from *E. coli* KS01 and JT1 by routine procedures (Sambrook *et al.*, 1989), and restriction enzymes, T4 DNA ligase and Klenow polymerase were used according to the manufacturer's instructions. Nucleotide sequences of the cloned fragments were determined using the dideoxy chain termination method (Sambrook *et al.*, 1989), [³⁵S]-dATP (Amersham, Buckinghamshire, UK) as a radioactive label, and a T7 DNA polymerase sequencing kit (Pharmacia Biotech., Uppsala, Sweden).

Construction of *E. coli* JT1 and verification of the mutation

The plasmid pMMS1 was constructed by cloning a 3 kb *SacI*–*KpnI* fragment from plasmid pPKL4 (Klemm, 1984), encoding the *fin* gene cluster of *E. coli* PC31, into the vector pBluescript II KS(+). A 1.4 kb *PstI* fragment carrying the *cat* gene of plasmid pACYC184 was cloned into a *PstI* site of the *finA* gene of plasmid pMMS1. The 4.4 kb fragment including *finA::cat* was inserted into the *pir*-dependent suicide plasmid pGP704 (Miller and Mekalanos, 1988) to create the plasmid pMSS3 used in homologous recombination with *E. coli* strain KS01. After selection on agar plates containing Cm and Tc, six transconjugants out of a total of 208 with the correct resistance characteristics (Ap^S, Km^S, Tc^R, Cm^R) were found. The phenotype of transconjugants were assessed by agglutination with yeast cells and in an antiserum against type-1 fimbriae of *E. coli* and by electron microscopy (Korhonen *et al.*, 1982; Saarela *et al.*, 1995). The correct genotype of the transconjugants was assessed by Southern hybridization of *Bam*HI, *Eco*RV and *Kpn*I digested chromosomal DNA (Sambrook *et al.*, 1989) with *finA* and *cat* as probes and using an enhanced-chemiluminescence kit (Amersham) for direct nucleic acid labelling and detection.

Amplification and cloning of the *fnbA* fragments into *fliC*_{H7Δ}

DNA fragments encoding one, two or three D repeats of staphylococcal FnBPA were amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase and the plasmid pFR015 (Flock *et al.*, 1987) as a template. pFR015 contains the *fnbA* gene of *S. aureus* in plasmid pUC18. The oligonucleotides used as primers contained an *AluI* site at the 5' end and were constructed on the basis of the nucleotide sequence of *fnbA* (Signäs *et al.*, 1989). The plasmid pWQ707 (Schoenhals and Whitfield, 1993), which contains the *fliC*_{H7} gene in vector pGEM-7Zf(+), was kindly provided by C. Whitfield (University of Guelph, Ontario, Canada). The 1755 bp coding region of *fliC*_{H7} was subcloned from pWQ707 as a *Xho*I–*Bam*HI fragment into the vector pBluescript II KS(+). A 174 bp *AccI* fragment in position 764–938 of the *fliC*_{H7} gene was removed, the cohesive ends were filled by Klenow polymerase and the *AluI*-digested *fnbA* fragments were blunt-end ligated to the deleted *fliC*_{H7} retaining the reading frame of both the *fliC* gene and the inserts. For expression, the *fliC::Tn10* gene of *E. coli* strain JT1 was complemented *in trans* with the various *fliC*_{H7}-containing plasmids.

PCR amplification and cloning of the *yadA* fragments into *fliC*_{H7Δ}

DNA fragments encoding YadA fragments of 48 (amino acid residues 84–131), 85 (84–168), 112 (274–385), 144 (131–274), 177 (26–202) or 302 (84–385) amino acids in length were amplified by PCR using as the template the plasmid pYMS4 (Skurnik and Wolf-Watz, 1989), carrying the *yadA* gene of *Y. enterocolitica* serotype O3 in pBR322. The oligonu-

cleotides used as primers included an *AccI* site at the 5' end and were constructed on the basis of the nucleotide sequence of *yadA* (Skurnik and Wolf-Watz, 1989). The DNA fragments were ligated into the *AccI*-digested *fliC_{H7Δ}* gene in pBluescript II KS(+) retaining the reading frame of both *fliC* and the *yadA* fragments.

Purification of chimeric flagella

For large-scale purification of flagella, bacteria from 300 agar plates were collected in 100 ml 10 mM Tris-HCl buffer, pH 7.5 (Tris) and flagella were sheared off the cells by a Turrax homogenizer for 45 s at 20 000 r.p.m. Cells were pelleted and the supernatant was recentrifuged twice to remove residual bacterial cells. Flagella in the supernatant were collected by ultracentrifugation (Ti45 rotor, 29 000 r.p.m., 3 h, 4°C). The flagella containing pellet was resolved in 1 ml 10 mM Tris-HCl, 0.5% deoxycholate, pH 7.5 (Tris-DOC) and flagella were purified by isopycnic ultracentrifugation (SW27 rotor, 20 000 r.p.m., 20 h, 4°C) in a 10–60% (w/w) sucrose gradient in Tris-DOC. The flagella were detected by illumination with concentrated light, collected and dialyzed against Tris and finally against distilled water. The filaments were concentrated in a rotavaporizer and the protein concentration was determined by a modified Lowry method. The purified flagella were analyzed by SDS-PAGE using a 5% (w/v) stacking gel and a 10% separating gel. Polypeptides visualized by Coomassie blue were transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus at 0.9 mA/cm² membrane for 2 h at 4°C. After transfer, the membrane was quenched with 2% bovine serum albumin (BSA) in phosphate-buffered saline, pH 7.1 (PBS) for 16 h at 20°C and washed with PBS. Polypeptides were visualized by staining with diluted polyclonal anti-flagella antibodies and alkaline phosphatase-conjugated secondary antibodies. A phosphatase substrate solution containing nitroblue-tetrazolium (162 µg/ml) and 5-bromo-4-chloro-3-indolyl-1-phosphate (370 µg/ml) was used.

In screening of chimeric flagella with the *YadA* inserts, we routinely used flagellar extracts that were obtained by vortexing for 5 min flagella-expressing cells from five agar plates in 500 µl PBS, the cells were then pelleted and the supernatant collected. In all binding tests, the *FliC* content in each extract and flagellar preparation was adjusted to 5 µg/ml after SDS-PAGE and analysis of the *FliC* peptide whole band intensity using image analysis (BioImage whole band software, BioImage, MI, USA) with an internal protein standard of known concentration.

Immunological methods and binding assays

Polyclonal antisera raised in rabbits against type-1 fimbriae of *E. coli* were available from previous work (Korhonen *et al.*, 1982), as was the monoclonal antibody 2G12 against *YadA* (Skurnik *et al.*, 1994). Polyclonal antibodies against H7 flagella were raised in rabbits by immunizing with purified H7 flagella from the *E. coli* strain IHE3034 *fimA::cat sfa::aad*; this strain will be described in detail elsewhere. Antibodies against the purified chimeric flagella were raised in rabbits, and the reactivity of the flagellar constructs with anti-H7 antibodies as well as with polyclonal anti-fibronectin antibodies (Vartio, 1982) were assessed by enzyme linked immunosorbent assays (ELISA) using routine procedures (Korhonen *et al.*, 1982).

In binding assays a modified ELISA assay was used. The purified chimeric flagella carrying D repeats of FnBPA were coated onto polystyrene 96-well microtiter plates at 0–5 µg *FliC*/ml for 16 h at 20°C. The wells were quenched with 2%

BSA/PBS for 1 h at 20°C and washed with PBS. Purified fibronectin from human plasma (Becton Dickinson, Bedford, MA, USA) or solubilized cellular fibronectin (Fibrogenex, Chigago, IL, USA) was added at 0–2.5 µg/ml in 0.1% BSA/PBS and 1 h later the wells were washed with PBS. Bound fibronectin was detected with diluted polyclonal anti-fibronectin antibodies and alkaline phosphatase-conjugated secondary antibodies. After addition of *p*-nitrophenyl phosphate substrate (1 mg/ml), the absorbance at 405 nm was measured in a Multiscan Titertek recorder. The assay was also performed *vice versa* by immobilizing plasma or cellular fibronectin (5.0 µg/ml) in microtiter wells. After quenching with 2% BSA/PBS, purified flagella (0–5.0 µg *FliC*/ml in 0.1% BSA/PBS) were added and after 1 h at 20°C, the wells were washed and bound flagella were detected with polyclonal anti-flagella antibodies and alkaline phosphatase-conjugated secondary antibodies. In tests with the chimeric purified flagella carrying *YadA* epitopes, type I or IV collagen (Sigma Co., St Louis, MO, USA) or BSA were coated onto a microtiter plate (Westerlund *et al.*, 1989), the wells were quenched as above and the flagella (5 µg/ml in 0.1% BSA/PBS) were added. After 2 h at 20°C, the wells were washed and bound flagella were detected as described above.

For immuno electron microscopy, bacterial cells expressing the various flagellar constructs were suspended in Luria broth and immobilized on copper grids coated with Pioloform and carbon. The bacteria were left to react with soluble fibronectin (100 µg/ml in 0.1% BSA/PBS) or with purified 30 kDa N-terminal fragment of fibronectin (250 µg/ml; Bional Ltd, Tartu, Estonia) or monoclonal antibody against *YadA* (diluted 1:10) for 1 h at 20°C. The grids were washed in 0.1% BSA/PBS and bound fibronectin was detected with polyclonal anti-fibronectin antibodies (diluted 1:300) and AuorprobeTMEM Protein A conjugate (Janssen Life Sciences Products, Olen, Belgium; diluted 1:40). Bound anti-*YadA* antibodies were detected with gold-conjugated anti-mouse antibodies (Dako-patts; diluted 1:25). Bacteria were negatively stained by 1% potassium tungstic acid (KPT), pH 7.0 for 30 s. The grids were examined in a Jeol JEM-100CX transmission electron microscope at an operating voltage of 60 kV.

In indirect immunofluorescence assays, cells of *S. aureus* DU5723 were suspended in PBS and immobilized on glass slides, fixed in 3.5% paraformaldehyde in PBS (PFA), washed and left to react with purified polyclonal immunoglobulin G (IgG) against *FliC_{H7Δ}/D1,D2,D3* flagella and *FliC_{H7Δ}* flagella (900 µg/ml). Bound IgG was detected with fluorescein isothiocyanate (FITC)-labelled secondary antibodies diluted 1:50. Stained bacteria were mounted in nicetamid and examined in an Olympus Standard fluorescence microscope (Olympus Optical Co., Hamburg, Germany) equipped with epi-illumination and interference filters for FITC.

Histological methods

Cryostat sections (5 µm thick) of human kidney were fixed and stained with purified flagella (60 µg/ml) essentially as described previously (Korhonen *et al.*, 1986). Bound flagella were detected with polyclonal anti-flagella antibodies, which were absorbed with human kidney homogenate for 16 h at 4°C to remove autofluorescence, diluted 1:10 and detected with FITC-labelled secondary antibodies diluted 1:50. Control sections were incubated similarly without flagella. In addition, some control sections were stained with a monoclonal antibody to cellular fibronectin diluted 1:100 and FITC-labelled anti-

mouse antibodies diluted 1:50. The nicetamid-mounted, stained sections were examined in the Olympus microscope.

Human embryonic skin fibroblasts (Hedman *et al.*, 1982) and human endothelial cells EA.hy926 (Edgell *et al.*, 1983) were grown to confluency on Lab-Tek Chamber slides (Nunc, Roskilde, Denmark). Cells were washed in PBS, fixed for 15 min at 20°C with PFA, washed again and incubated with purified flagella (35 µg/ml in PBS) for 1 h at 20°C. Bound flagella were detected by preabsorbed anti-flagella antibodies (diluted 1:10) and FITC-labelled secondary antibodies (diluted 1:50). Control cell cultures were incubated similarly but in the absence of flagella. For localization of secreted fibronectin, cells were also stained with a monoclonal anti-fibronectin antibody (Chemicon International Inc., CA, USA; diluted 1:100) and FITC-labelled secondary antibodies (diluted 1:50). The cell cultures were mounted and examined in the Olympus microscope.

Adhesion of *S.aureus* DU5723 to immobilized fibronectin

Glass slides were coated with purified plasma fibronectin (10 µg/ml) for 16 h at 20°C and quenched in 2% BSA/PBS for 2 h at 20°C. Protein A-deficient *S.aureus* DU5723 cells (1.75×10^8 cells/ml) expressing FnBPA were incubated for 30 min at 20°C in the presence of purified anti-flagella IgG (anti-FliC_{H7Δ}/D1,D2,D3 and anti-FliC_{H7Δ} flagella antibodies) at a concentration of 900 µg/ml. Control wells were incubated similarly with bacteria incubated in the absence of antibodies. Bacteria were then allowed to adhere to immobilized fibronectin for 2 h at 20°C. The glasses were washed and adherent bacteria visualized by staining with methylene blue. The number of adhered bacteria in 20 microscopic fields of 4.8×10^3 mm² was calculated.

Results

Construction of the non-adhesive host strain *E.coli* JT1

The flagella-deficient strain *E.coli* C600 *hsm hsr fliC::Tn10* (also called *E.coli* KS01; Kuwajima, 1988a) carries a silenced *fliC* gene but has the other genes needed for the synthesis and polymerization of functional flagellar filaments. The strain also expresses the common, mannoside-binding type-1 fimbriae, and we therefore silenced the *fimA* gene using allelic replacement. Transconjugants with correct antibiotic markers were assessed by electron microscopy and by agglutinations with yeast cells as well as in an antiserum against *E.coli* type-1 fimbriae. A transconjugant exhibiting no fimbriae was chosen and termed *E.coli* JT1. The correct genotype of JT1 was confirmed by Southern hybridizations (data not shown).

Construction and characterization of chimeric flagella expressing repeats of the *S.aureus* FnBPA

The complete coding region of the *fliC*_{H7} gene was subcloned and a 174 bp *AccI* fragment in the variable region of the *fliC*_{H7} gene was deleted. DNA fragments encoding D3 (117 bp), D2, D3 (231 bp) or D1, D2, D3 (345 bp) repeats of FnBPA were amplified by PCR and inserted in the *AccI* site while retaining the reading frame of *fliC* and the inserts. The correct nucleotide sequence of each D insert was verified by nucleotide sequencing using as primers the *fliC*_{H7} sequences downstream and upstream of the cloning site. Complementation *in trans* of *fliC::Tn10* of strain JT1 with each of the *fliC* constructs resulted in expression of flagella with normal morphology. Each of the resulting recombinant *E.coli* strains were also motile.

The flagella purified from each construct were analysed by

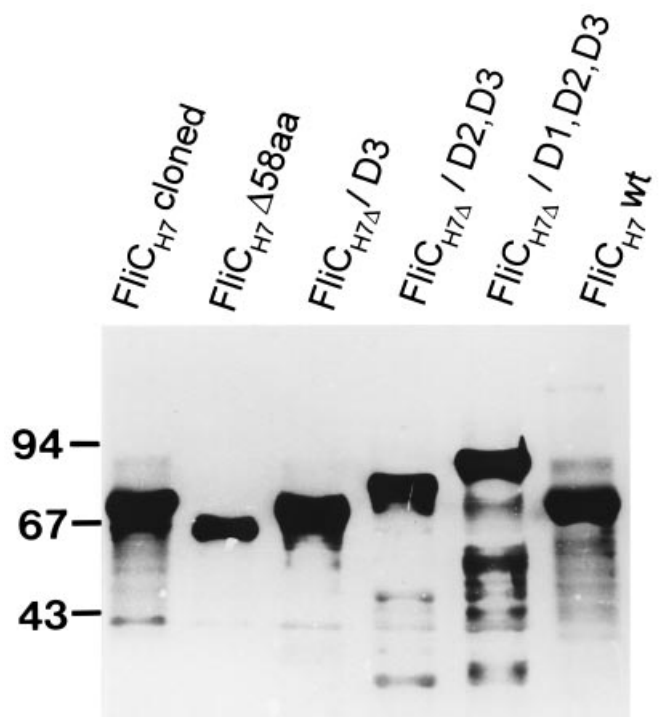


Fig. 1. Western blot analysis with polyclonal anti-H7-flagella antibodies of the purified flagella. Lanes from left to right: H7 flagella carrying intact flagellins and purified from a recombinant strain (FliC_{H7} cloned), flagella lacking 58 amino acids in the variable region of the flagellin (FliC_{H7Δ}58aa), flagella carrying one fibronectin-binding repeat of FnBPA per flagellin (FliC_{H7Δ}/D3), flagella carrying two repeats per flagellin (FliC_{H7Δ}/D2,D3), flagella carrying three repeats per flagellin (FliC_{H7Δ}/D1,D2,D3), and H7 flagella purified from a wild-type strain (FliC_{H7}wt). The position of molecular weight markers in kDa are indicated on the left.

SDS-PAGE (not shown) and by Western blotting (Figure 1) using polyclonal anti-H7 antibodies. The major polypeptides in the preparations were FliC monomers strongly reacting with the anti-H7 antibodies in the Western blots. The apparent size of the flagellins corresponded to those predicted from the nucleotide sequences, i.e. it increased with the size of the insert in *fliC*. The polypeptides of smaller apparent size that were present in the preparations and also reacted with the antibodies most likely were flagellar minor proteins and FlgE forming the flagellar hook (see Figure 3 below). As the subsequent binding tests were based on an immunological detection with either polyclonal anti-H7 antibodies or polyclonal anti-fibronectin antibodies, we measured their reactivity with the flagellar constructs by ELISA. The anti-fibronectin antibodies gave no detectable reaction with the flagella, and the anti-H7 antibodies reacted similarly with the chimeric flagella and the deletion derivative (data not shown).

Next we tested the fibronectin-binding properties of the flagellar constructs. Purified flagella were immobilized onto microtiter wells and allowed to react with purified, soluble plasma fibronectin (Figure 2) or solubilized cellular fibronectin (data not shown). The binding of fibronectin by chimeric flagella was dose-dependent, saturable (Figure 2A and B) and equally strong with plasma and cellular fibronectin (not shown). The binding was most efficient with the flagella carrying three fibronectin-binding inserts (FliC_{H7Δ}/D1,D2,D3) and least efficient with the flagella expressing only the D3 repeat (FliC_{H7Δ}/D3). Flagella lacking inserts (FliC_{H7Δ}) did not bind

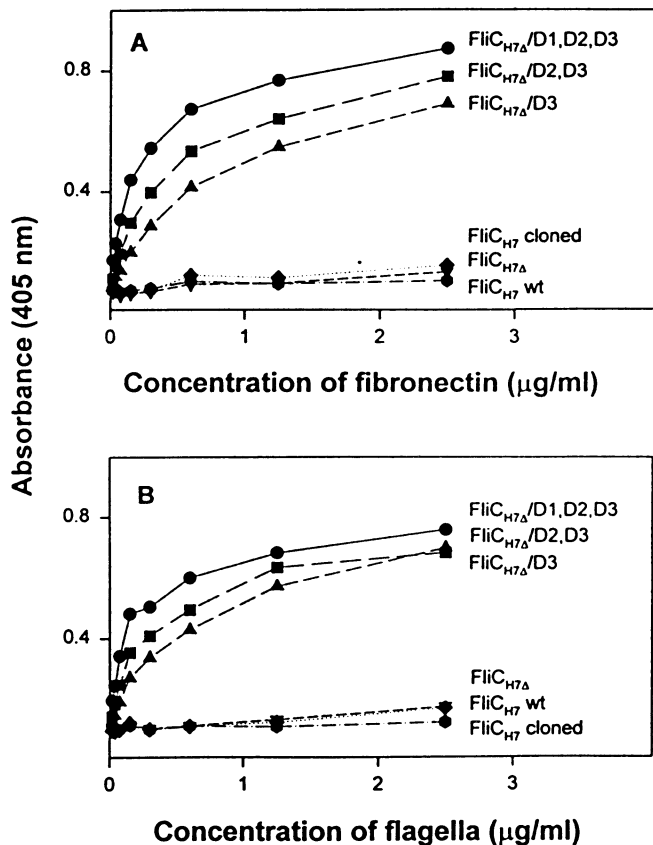


Fig. 2. Binding of immobilized purified flagella to soluble plasma fibronectin analysed by ELISA. The flagellar constructs were immobilized on microtiter wells at a constant concentration of 2.5 µg of FliC/ml (A) and at an increasing concentration of 0–2.5 µg of FliC/ml (B). Soluble fibronectin was added at 0–2.5 µg/ml (A) and 2.5 µg/ml (B), and the binding was detected using anti-fibronectin antibodies and phosphatase-conjugated secondary antibodies.

fibronectin. These assays were also performed *vice versa*, i.e. by immobilizing plasma fibronectin and testing the binding of soluble flagella; essentially the same results were obtained (data not shown).

We also visualized the fibronectin binding by immuno electron microscopy. No binding of fibronectin to the FliC_{H7Δ} flagella was observed (Figure 3A), whereas a deposit of fibronectin was observed along the FliC_{H7Δ}/D3 filament (Figure 3B). The FliC_{H7Δ}/D1,D2,D3 filament had a thicker deposit of fibronectin (Figure 3C). The deposition of the N-terminal fragment of fibronectin on FliC_{H7Δ}/D1,D2,D3 flagella was visible also in a direct negative staining without antibodies (Figure 3E). The specificity to FliC of the fibronectin-binding property of the chimeric flagella can be seen in Figure 3D, which shows a strong binding to the *fliC*-encoded filament and a lack of binding by the flagellar hooks encoded by the *flgE* gene.

To assess the potential of the chimeric flagella in histological detection and localization of receptor-active tissue domains, we tested the binding of the chimeric flagella to frozen sections of human kidney, a histologically well characterized tissue. The tissue sections were immunostained with a monoclonal antibody specific to the cellular fibronectin (Figure 4A) as well as with the chimeric (Figure 4B) or the FliC_{H7Δ} (Figure 4C) flagella. A colocalization of the binding site of the antibody and of the FliC_{H7Δ}/D1,D2,D3 flagella to glomerular mesangial

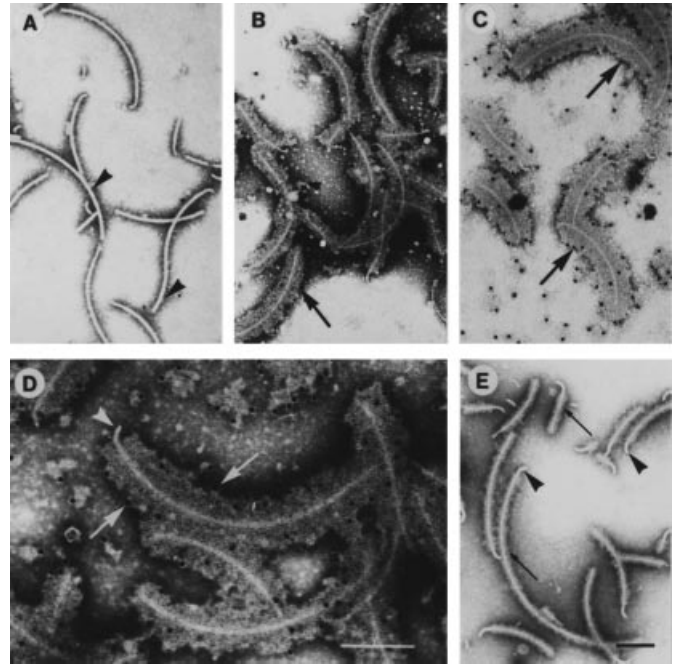


Fig. 3. Immunogold electron microscopy analyses of flagella incubated with soluble fibronectin (A to D) and with the 30 kDa N-terminal fragment of fibronectin (E). (A) The FliC_{H7Δ} flagella, (B) the FliC_{H7Δ}/D3 flagella, (C) the FliC_{H7Δ}/D1,D2,D3 flagella, and (D) the FliC_{H7Δ}/D2,D3 flagella incubated with soluble fibronectin. (E) The FliC_{H7Δ}/D1,D2,D3 flagella incubated with 30 kDa N-terminal fragment of fibronectin. The binding was visualized with anti-fibronectin and Protein A-gold in (A–D), whereas in (E) staining was without antibodies. Arrows indicate deposition of fibronectin, arrowheads indicate lack of binding. Size bars, 200 nm.

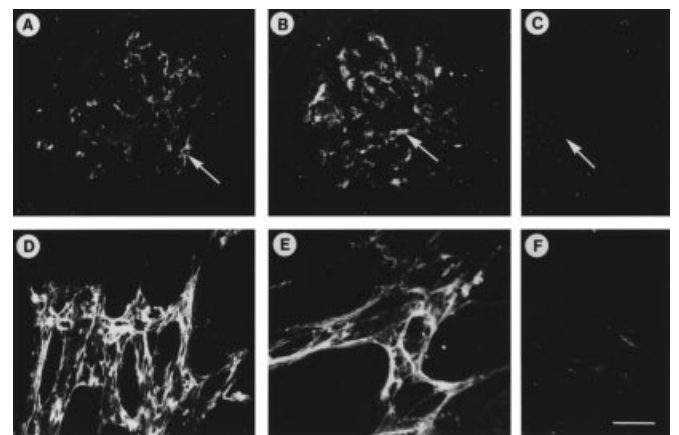


Fig. 4. Binding of the chimeric flagella and the monoclonal anti-fibronectin antibody to frozen sections of human kidney (A–C) and to cultured human embryonic skin fibroblasts (D–F) visualized by indirect immunofluorescence. Binding of (A, D) the antibody, (B, E) the FliC_{H7Δ}/D1,D2,D3 flagella, and (C, F) the FliC_{H7Δ} flagella are shown. Arrows in (A–C) indicate glomerular mesangium. Bar represents 30 µm.

areas was evident; no binding to kidney tissue was seen with the FliC_{H7Δ} flagella. Again, the FliC_{H7Δ}/D1,D2,D3 flagella bound more strongly to the tissue sections than did the FliC_{H7Δ}/D3 or the FliC_{H7Δ}/D2,D3 flagella (data not shown).

A staining of human embryonic skin fibroblasts (Figure 4D–F) which express fibronectin abundantly (Hedman *et al.*, 1982), and malignant human endothelial cells expressing fibronectin poorly (Kreis and Vale, 1993), revealed highly similar binding patterns by the chimeric flagella and the anti-fibronectin antibody (results for fibroblasts shown in Figure 4D, E and F).

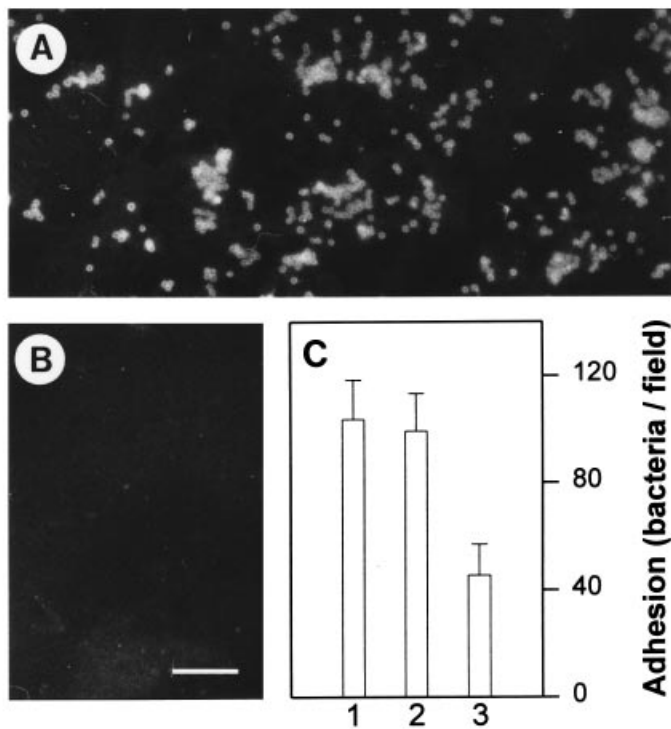


Fig. 5. Indirect immunofluorescence staining of the Protein A-deficient *S. aureus* strain DU5723 with anti-flagella antibodies (A and B) and adhesion of *S. aureus* DU5723 to immobilized fibronectin in the presence of anti-flagella antibodies (C). The staining with anti-FliC_{H7Δ}/D1,D2,D3 flagella antibodies is shown in (A), and with anti-FliC_{H7Δ} flagella antibodies in (B). (C) Adhesion to fibronectin by *S. aureus* DU5723 in the absence of antibodies (bar 1) and in the presence of the anti-FliC_{H7Δ} (bar 2) and the anti-FliC_{H7Δ}/D1,D2,D3 immunoglobulins (bar 3) are shown. Means and standard deviations of adherent bacteria in 20 microscopic fields of $4.8 \times 10^3 \mu\text{m}^2$ are shown. Size bar, 10 μm .

Anti-adhesive antibodies

We raised rabbit antibodies against flagellar constructs carrying D repeats and tested in an indirect immunofluorescence assay the binding of the antibodies to a Protein A-deficient strain of *S. aureus* (DU5723) expressing the FnBPA protein. Antibodies against the FliC_{H7Δ}/D1,D2,D3 flagella bound efficiently to the bacterial cells (Figure 5A), no binding was seen with the anti-FliC_{H7Δ} flagella antibodies (Figure 5B). The antibodies against FliC_{H7Δ}/D1,D2,D3 also inhibited the adhesion of DU5723 to fibronectin (Figure 5C).

Binding characteristics of the chimeric flagella carrying fragments of YadA

The YadA peptides expressed as fusions to FliC are schematically presented in Figure 6A. The deletion of amino acid residues 83–104 (Tamm *et al.*, 1993) as well as of the point mutations in the sequence HSSH at the position 159–162 (Roggenkamp *et al.*, 1994), both reported to affect collagen binding, also are indicated in Figure 6A. The C-terminal region at residues 385–455 influences export and assembly of YadA polymers at the bacterial surface (Tamm *et al.*, 1993).

Binding of the YadA–FliC fusion proteins to type I and IV collagens is shown in Figure 6B. Only the flagella with the longest YadA insert (302 amino acids; residues 84–385) bound to collagens; the binding of the other construct was negligible and close to the level seen with the FliC_{H7Δ} flagella. The YadA 84–385/FliC flagella also reacted with the monoclonal anti-

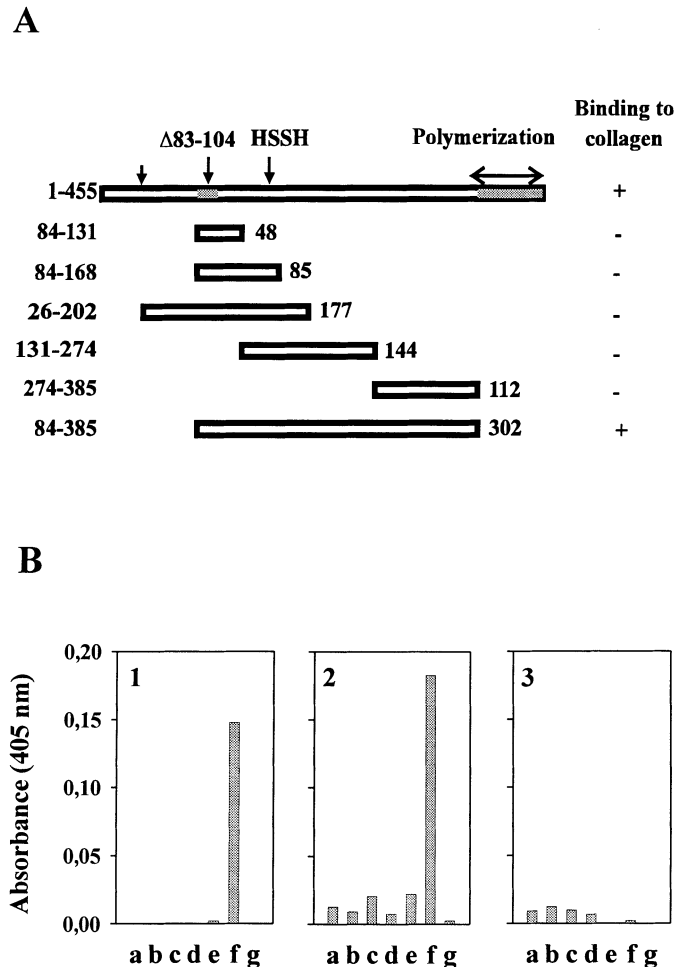


Fig. 6. Binding of YadA/FliC fusions to collagens. (A) Schematic presentation of the YadA fragments expressed as fusions to FliC are shown. On top, the entire 455 amino acid YadA peptide. The cleavage site for signal peptide is indicated by a small arrow. The two mutation sites ($\Delta 83$ –104; HSSH) reported to affect collagen binding as well as the C-terminal region affecting polymerization and surface localization are also shown. Shaded areas indicate predicted hydrophobic regions. The number after the fragment gives the size of the insert in amino acids. (B) Binding of the YadA/FliC constructs to immobilized (1) type IV collagen; (2) type I collagen; and (3) BSA analyzed by a modified ELISA. The flagella were (a) YadA 84–131/FliC; (b) YadA 84–168/FliC; (c) YadA 26–202/FliC; (d) YadA 131–274/FliC; (e) YadA 274–385/FliC; (f) YadA 84–385/FliC; and (g) FliC_{H7Δ}.

YadA antibody 2G12 (details not shown). Bacterial cells expressing each of the YadA/FliC construct were motile.

Discussion

Earlier studies (Kuwajima, 1988a; 1988b; Newton *et al.*, 1989) have shown that large fragments from the variable domain of flagellins can be deleted and that short foreign peptides can be expressed at this site in *E. coli* and *Salmonella* flagellins without gross effects on the biogenesis and motility of flagella. These studies were aimed at the construction of chimeric vaccines, and the inserted antigenic fragments have been fairly short, in the range of 15–26 amino acids. While our study was in progress, Lu *et al.* (1995) reported the construction of a random peptide library based on *E. coli* FliC. In their system, random dodecapeptides were expressed in *E. coli* thioredoxin fused into the variable region of FliC and used by biopanning to identify antigenic epitopes recognized by monoclonal anti-

bodies. Our results extend the use of FliC technology beyond immunology in demonstrating that adhesive peptides, ranging in size between 39 and 302 amino acids in size, can be functionally expressed as fusions in FliC. It has been considered that bacterial surface appendages or outer membrane proteins are not suitable carriers for display of large (i.e. >60mer) polypeptides (reviewed by Georgiou *et al.*, 1997). We also show that chimeric flagella can be used in histological localization of tissue targets for a defined adhesive epitope. We constructed the expression system in the *E.coli* strain JT1 which we mutated for the expression of the common, mannoside-binding type-1 fimbriae, to overcome addition of mannoses to the binding assays.

We used a 58 amino acid deletion in FliC as the site to express the adhesive epitopes. The resulting chimeric flagella retained filamentous morphology and supported bacterial motility, indicating that the flagellar filament tolerates large insertions in the variable domain to remain functional. The size of the insert in FliC most likely is not restricted to the 302mer insert expressed in this study. Kuwajima (1988b) found that flagellar biogenesis and motility tolerate a deletion of 187 amino acid residues in the variable region of FliC, and hence use of larger (>58mer) deletions should allow expression of larger (>302mer) inserts when this is needed. Bacterial FliC sequences do not contain cysteine residues (Wilson and Beveridge, 1993), and the assembly machinery of flagella bypasses the periplasmic space of *E.coli* where disulphide bonds are formed. Lack or low content of cysteine residues is typical for surface proteins of Gram-positive bacteria and for bacterial S-layers, i.e. the types of adhesion proteins used in this study, as well as for those surface proteins of Gram-negative bacteria that are secreted by the type III secretion system (Salmond, 1994). Our experience in expressing in FliC peptides from fimbrial adhesins, which infrequently contain disulphide bonds, has shown that the fusion flagella are synthesized but the cysteine thiol groups need to be oxidized to disulphide bonds *in vitro* in the isolated chimeric flagellar filament in order to obtain functional insertions (J.Tanskanen, unpublished data). Correct *in vitro* folding and oxidation obviously will be a case-to-case property of the inserted peptide, and this may be a limitation to the use of FliC technology in adhesion studies. On the other hand, Lu *et al.* (1995) reported expression of dodecapeptides in a thioredoxin active site loop fused in FliC; the FLITRX technology can express 20mer inserts assumed to be displayed as conformationally constrained structures in a disulphide loop of the fused thioredoxin peptide. However, the presence of disulphide bonds in the FLITRX flagella was not physically documented.

We demonstrated that the chimeric flagella with the D repeats specifically bound to soluble and immobilized human plasma and cellular fibronectin as well as to renal mesangium and cultured fibroblasts rich in cellular fibronectin. The FliC_{H7Δ}/D1,D2,D3 flagella bound to the fibronectin-containing targets with a higher affinity than did the FliC_{H7Δ}/D2,D3 or the FliC_{H7Δ}/D3 flagella. The targets for the D repeats in the N-terminal part of human plasma fibronectin are the type I repeats (Scottile *et al.*, 1991), and we also demonstrated binding of the N-terminal fragment of fibronectin to the chimeric flagella. The high affinity of FliC_{H7Δ}/D1,D2,D3 to fibronectin may result from a simultaneous binding of three D repeats to adjacent target repeats in fibronectin. A conclusion from our work is that the triplicate D repeats have evolved in *S.aureus* to increase the affinity of FnBPA to fibronectin, which suggests

that fibronectin binding serves an important colonization function for *S.aureus*. Our results also demonstrate that the D repeats recognize the cellular form of fibronectin.

We applied the flagella display to the less well characterized adhesin YadA of *Yersinia*. Expression of the YadA regions carrying the two previously reported mutations affecting collagen binding (see Figure 6), separately or in combination in short (up to 177mer) fusions did not confer collagen binding. The construct YadA 84–385 was the only one reacting with collagens. This indicates that the collagen binding site in YadA is non-linear and also contains regions C-terminal to the previously identified sites. Another explanation is that correct conformation of a putative linear collagen binding epitope is influenced by large portion of the YadA molecule. The resolution of this problem requires knowledge of the three-dimensional structure of YadA. However, indirect evidence from inhibition studies with a synthetic peptide mimicking the HSSH region (Roggenkamp *et al.*, 1995) and with poly- and monoclonal anti-YadA antibodies (Y.el Tahir and M.Skurnik, unpublished data) are in accordance with our hypothesis of a non-linear collagen-binding epitope.

We believe the chimeric flagella expressing bacterial adhesive epitopes offer various important advancements in adhesion technology. Flagella are easy to produce and purify in large quantities and tolerate large insertions in FliC. In certain applications, such as in studies of cell responses to an adhesive epitope, use of purified flagella instead of bacterial cells is desirable due, for example, to the presence of the reactive endotoxin on the bacterial cell surface. The main interest in our study was to construct fusion proteins suitable for molecular studies on adhesin–receptor interactions of pathogenic bacteria. Epithelial receptor density for a given bacterial adhesive epitope varies between individuals (Rutter and Jones, 1973; Källénus and Winberg, 1978), according to host age, as well as in different tissue domains (Runnels *et al.*, 1980; Korhonen *et al.*, 1990) and is a primary determinant of the susceptibility of certain individuals to various infectious diseases. An early identification of those individuals who express a high receptor density and thus are prone to repeated infections would offer possibilities for preventive measures by vaccination or other means. Our demonstration that the chimeric flagella bind to frozen human tissue sections and to cultured epithelial cells in a manner similar to an anti-fibronectin antibody exemplifies that such flagella can be applied to localize and quantitate receptor–active tissue domains for a bacterial adhesion epitope. Such tools are particularly important in the study of tissue receptors for bacterial adhesins, as it is evident that a given tissue receptor molecule is recognized differently by bacteria and by plant lectins or antibodies (Karlsson, 1989). The multivalency of the chimeric flagella leads to high affinity ligands that could be effective in, for example, identification of receptors by Western blotting, isolation of receptors from tissue homogenates as well as in studying human cell responses to a particular adhesive epitope. Fusion of a large fragment of the hydrophobic YadA protein resulted in flagella that were functional and remained in suspension, demonstrating that flagella display offers a tool for the study of S-layer type of hydrophobic adhesins.

Anti-adhesive antibodies are a focus of current research aiming at preventive measures against bacterial and viral infections. We found that immunization with the chimeric flagella evoked antibodies which bound to *S.aureus* cells and inhibited their adhesiveness to fibronectin. The observed

inhibition by the immunoglobulins against FliC_{H7Δ}/D1,D2,D3 flagella was only partial. This was to be expected as *S.aureus* DU5723 contains multiple fibronectin-binding proteins (FnBPA, FnBPB) and as FnBPA has a further fibronectin-binding site (termed CB) outside the D-repeat region (Jönsson *et al.*, 1991). Chimeric flagella express the inserts in thousands of copies along the filament surface and are effective immunogens. Our results encourage further design of chimeric flagella as tools to raise specific anti-adhesive antibodies as well as to identify and characterize adhesion proteins and their tissue targets.

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