Simultaneous Display of Multiple Foreign Peptides in the FliD Capping and FliC Filament Proteins of the Escherichia coli Flagellum

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Received 16 December 2004/Accepted 3 March 2005

The bacterial flagellum is composed of more than 20 different proteins. The filament, which constitutes the major extracellular part of the flagellum, is built up of approximately 20,000 FliC molecules that assemble at the growing distal end of the filament. A capping structure composed of five FliD molecules located at the tip of the filament promotes polymerization of FliC. Lack of FliD leads to release of the subunits into the growth medium. We show here that FliD can be successfully used in bacterial surface display. We tested various insertion sites in the capping protein, and the optimal region for display was at the variable region in FliD. Deletion and/or insertion at other sites resulted in decreased formation of flagella. We further developed the technique into a multihybrid display system in which three foreign peptides are simultaneously expressed within the same flagellum, i.e., D repeats of FnBPA from Staphylococcus aureus at the tip and fragments of YadA from Yersinia enterocolitica as well as SlpA from Lactobacillus crispatus along the filament. This technology can have biotechnological applications, e.g., in simultaneous delivery of several effector molecules.

The flagellum is the bacterial motility organelle and is composed of more than 20 different proteins that build up the basal body, the hook, the filament, and the flagellum-specific type III secretion system. The organelle traverses the bacterial inner and outer membranes and forms a 2-nm-wide central channel, inside which the FliC flagellin subunits are transported to the growing distal end, where they assemble to a filament of approximately 20,000 FliC copies. Flagellar assembly is strictly regulated by hierarchical expression of the genes encoding flagellar components and proceeds in a highly ordered fashion from the basal body components and the secretion machinery towards the hook and the filament (11).

The capping protein FliD (also called HAP2) has a central role in flagellar polymerization. After completion of the synthesis of hook-basal body complex, the FliD cap is attached to the tip of the hook and newly exported FliC monomers insert between the hook and the cap. The cap is essential for polymerization of FliC monomers as well as assembly and growth of the flagellar filament. Deletion of fliD leads to inability of transported FliC to polymerize into filaments and to their secretion into the growing medium (6, 8). A high-resolution map based on electron microscopy revealed that five FliD molecules assemble to form the stool-like flagellar cap that consists of five leg-like domains and a plate-like structure (Fig. 1). The legs anchor the cap to the tip of the flagellar filament, whereas the plate forms a lid on the top of the filament (14, 28). The highly conserved N-terminal 40 amino acids and C-terminal 50 residues of FliD are located in the leg domain embedded at the cap-filament interface and play an essential role in capping activity and in stabilizing the cap-filament interaction. The central part of FliD that builds up the cap plate is important for the polymerization of FliD (13, 14, 25, 28).

Bacterial surface display has been successfully used, as a complement to phage display, in vaccine development, studies on protein-protein interactions, as bioadsorbents, and in construction of synthetic random peptide libraries (17) and recently also to identify peptides that mediate microbial entry into eukaryotic cells (23). Flagellum display is based on gene fusion of foreign DNA to a variable part of flic and has been used in, e.g., presentation of short antigenic determinants, production of recombinant vaccines, and characterization of bacterial adhesive peptides (10, 16, 26). The technique allows a multivalent display system that tolerates insertion of large foreign peptides on the surface of bacteria.

We have earlier shown that adhesive peptides up to 302 amino acids in length can be displayed in a functional form along the flagellar filament as fusions to the variable region of FliC (27) and that the flagellar filament allows simultaneous multivalent display of two different inserts fused to the variable region of FliC (22). Here we examined the applicability of the capping protein FliD for display purposes and further developed the flagellum display into a multihybrid surface display system in which three different foreign peptides are expressed simultaneously within the same flagellum, i.e., at the tip and along the filament. As foreign inserts, we used the fibronecrtin-binding D repeats of FnBPA from Staphylococcus aureus (19) and the collagen-binding YadA adhesin from Yersinia enterocolitica (21), which originate from the virulence factors of important human-pathogenic bacteria and/or represent potential vaccine candidates, as well as the SlpA S-layer protein of Lactobacillus brevis (7).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Escherichia coli strain MG1655 ΔfimA-H (recA Sm Rif), also called AAEC072A (3), was used for construction of the fliD mutant strain MKS1 and flic mutant strain MKS2. MKS2 was used for

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FIG. 1. Schematic presentation of the flagellar cap based on the three-dimensional image of *Salmonella* FliD (28). Five FliD molecules assemble to form a plate-like structure and five leg-like domains. The plate-like domain is surface exposed, whereas the leg-like domains are plugged into the cavity at the tip of the flagellar filament. Each leg is formed by one FliD molecule and contains the conserved, structurally disordered N and C termini, indicated by hatching (25, 28).

FIG. 2. *E. coli* strains and their genotypes and plasmids constructed in the study. The efficiency of flagellar formation in the recombinant strains is also shown. The sequences of the plasmid vectors pTRC99A, pACYC184, and pBluescript KSII(−) are represented by solid, dashed, and dotted lines, respectively, from the chromosome of *E. coli* strain S17-1 pir (recA thi pro hsdR M’ RP4-2-Tc::Mu::Km Tn7, λ pir lysogen; Sm’ Tp’). (20) was used as a donor in conjugation for constructing strains MKS1, MKS2, and MKSD3. *E. coli* strain XL1-Blue MRF+(Stratagene, La Jolla, CA) was used for cloning purposes.

For immunogold electron microscopy analyses, the strains expressing hybrid FliD and MKS2 (pD1D2D3/FliC17A) were grown on Luria agar or statically in 3 ml of Luria broth for 18 h at 37°C. Strain MKSD3 (pSlpA96-245/FliC17A) (pYadA84-385/FliC17A) was grown on Luria agar for 3 days at 28°C. For preparation of flagellar crude extracts the strains were grown on Luria agar as described above. For other purposes bacteria were grown with shaking in Luria broth for 16 h at 37°C. Media were supplemented with ampicillin at 100\(\mu\)g/ml, rifampin at 75\(\mu\)g/ml, or streptomycin at 100\(\mu\)g/ml, when appropriate.

![Construction of the mutant strains.](https://example.com) The mutant strains were constructed by site-specific mutagenesis using the pir-dependent suicide vector pCVD442 containing the *sbcE* gene of *Bacillus subtilis* (5) as essentially as described (15). Strains MKS1 and MKS2 were obtained by site-specific deletion of *fliD* and *fliC*, respectively, from the chromosome of *E. coli* MG1655 Δ*fimA*-H. A 2,248-bp fragment carrying a deletion of nucleotides 186 to 1214 in *fliD* and a 2,482-bp fragment carrying a deletion of nucleotides 191 to 1345 in *fliC* were generated by PCR using chromosomal DNA of MG1655 Δ*fimA*-H as the template. The primers were designed on the basis of the nucleotide sequence of MG1655 (2). Mutant MKSD3 was constructed by site-specific insertion of the Δ*fliD*-D3 fragment into *fliD* in MKS2. The Δ*fliD*-D3 fragment was PCR amplified using pD3/FliΔD3 as the template. Correct genotypes of the mutants were confirmed by PCR and Southern hybridization, and phenotypes by electron microscopy and colony dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis.

**Plasmid constructions.** The plasmids used are shown in Fig. 2. All the gene fragments were cloned in frame to retain the reading frame of the gene fusions. Correct cloning was confirmed by restriction mapping or nucleotide sequencing. In plasmid pFliΔD3, an additional SacI restriction site was constructed by PCR into the deletion site. Plasmids SlpA96-245/FliC17A and YadA84-385/FliC17A encoding hybrid flagellins were available from previous studies (7, 27). For this study the gene encoding YadA84-385/FliC17A was cloned into pACYC184 (New England Biolabs, Beverly, MA) to obtain pYadA84-385/FliC17A, which is compatible with pSlpA96-245/FliC17A.

**Proteins and antisera used.** Human plasma fibronectin was from Collaborative Medical Products (Bedford, MA). Anti-fibronectin antibodies and antibodies against the His-tagged D1-D3 peptide available from previous work (12) were raised in rabbits according to standard immunization protocols (27). Anti-SlpA96-245 antibodies were available from previous work (7, 27). The monoclonal anti-YadA antibody was a kind gift from M. Skurnik (University of Helsinki). Colloidal gold-conjugated anti-mouse and anti-rabbit antibodies were from BSA International Ltd. (Cardiff, United Kingdom) and protein A-gold was from Amersham Biosciences (Piscataway, NJ).

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<th>Strain</th>
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<td>MKS1 Δ<em>fliD</em></td>
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<td>MKS1 Δ<em>fliD</em>Δ14</td>
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<td>MKS1 Δ<em>fliD</em>Δ39</td>
<td>pSlpA96-245/FliΔ14</td>
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<td>MKS1 Δ<em>fliD</em>Δ39</td>
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<td>MKS2 Δ<em>fliC</em></td>
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<td>MKS2 Δ<em>fliC</em>ΔfliD*Δ39</td>
<td>pFliC*MG1655</td>
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<td>MKSD3 Δ<em>fliC</em>ΔfliD*Δ39</td>
<td>pYadA84-385/FliC*17A</td>
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<td>MKSD3 Δ<em>fliC</em>ΔfliD*Δ39</td>
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The symbols +, ++, and +++ indicate weak, intermediate, and wild-type formation of flagella assessed by SDS-PAGE analysis and electron microscopy, respectively; see Fig. 4 for examples.
Analysis of flagellar filament formation by SDS-PAGE. Flagellar crude extracts were prepared as described before (27). Briefly, cells were collected from Luria agar plates and vigorously suspended in 50 μl phosphate-buffered saline, pH 7.1. The cells were pelleted, the supernatants were centrifuged once more, and 10 μl of the cell-free sample was loaded onto the SDS-PAGE gel.

Immunoelectron microscopy analysis. For immunoelectron microscopy analysis bacteria were suspended in 50 μl LB medium and immobilized on nickel grids coated with Pioloform and carbon. The immunostainings were done with antibodies, and colloidal gold-conjugated protein A for visualization (Fig. 5A, panel a). For comparison, fibronectin binding by flagella of E. coli MKS2(pD1D2D3/FliC_{H7}) expressing D repeats as FliC fusions along the filament side is shown in Fig. 5A, panel c. The fibronectin deletion derivative E. coli MKS2 was constructed and used to avoid expression of wild-type FliC.

RESULTS

Display of foreign peptides in FliD and FliC. Alignment of FliD protein sequences revealed three short variable sequences, which were tested as insertion sites for foreign peptides by gene fusion (Fig. 3). We constructed the plasmid pFliD by PCR cloning the fliD gene of E. coli MG1655 ΔfimA-H into the vector pTrc99A. We then deleted separately the regions corresponding to amino acids 205 to 219 and 254 to 263 of the vector pTrc99A. We then deleted separately the regions corresponding to amino acids 205 to 219 and 254 to 263 of the vector pTrc99A. The N and C termini of FliD are indicated. D1, D2, and D3 refer to D repeats from S. aureus, and SlpA refers to the N-terminal fragment of SlpA from L. brevis.

As a different type of peptide, we next inserted into FliD an epitheial cell-binding N-terminal fragment (150 amino acids) of the S-layer protein SlpA from Lactobacillus brevis (Fig. 3). When plasmid pSlpA96-245/FliD was used to complement the ΔfliD gene in E. coli MKS1, the strain expressed flagella almost at the same level as did E. coli MKS1(pFliDΔΔ14) expressing D repeats in fliD as FliC fusions along the filament side (data not shown). Finally, the D1 repeat was inserted at amino acid 151, reduced compared to wild-type filament formation (data not shown). The resulting plasmid, pFliD_{D1FliC_{H7}} (Fig. 2), did not complement the ΔfliD gene in E. coli MKS1 (data not shown). The results indicated that cloning of D repeat gene fragments into a deletion site in fliD results in more efficient expression of hybrid FliD than insertion into an existing restriction site. Thus, we proceeded with the plasmid pFliDΔ14.

We cloned a 345-bp fragment encoding the fibronectin-binding D1, D2, and D3 repeats (115 amino acids) from S. aureus (19) into pFliDΔ14 (Fig. 2 and 3). The resulting strain, E. coli MKS1(pD1D2D3/FliD_{C14}) formed flagellar filaments at a considerably lower level than the wild-type strain but expressed fibronectin-binding D repeats at the filament tip (data not shown).

A different type of peptide, we next inserted into FliD an epitheial cell-binding N-terminal fragment (150 amino acids) of the S-layer protein SlpA (7) of Lactobacillus brevis (Fig. 3). When plasmid pSlpA96-245/FliDΔ14 was used to complement the ΔfliD gene in E. coli MKS1, the strain expressed flagella almost at the same level as did E. coli MKS1(pFliDΔΔ14) expressing D repeats in fliD as FliC fusions along the filament side (data not shown). The flagella of E. coli MKS1 (pSlpA96-245/FliDΔ14) carried hybrid SlpA/ΔFliD at the tip, as shown by immunoelectron microscopy using anti-SlpA antibodies and colloidal gold-conjugated protein A (Fig. 5A, panel e). The flagella of the control strain MKSD2(pFliC_{H7}) did not interact with anti-SlpA antibodies (Fig. 5A, panel f).

Trihybrid display of peptides along the filament and at the filament tip. For simultaneous expression of heterologous peptides along the filament and at the tip we first constructed the E. coli mutant strain MKSD3. The fliD gene of MKS2 was

![FIG. 4. Efficiency of flagellar formation by the various E. coli recombinant strains: SDS-PAGE analysis of flagellar crude extracts obtained from the strains indicated above the gel. The major polypeptide visible corresponds to FliC. The symbols −, +, ++, and +++ indicate no, weak, intermediate, and wild-type formation of flagellar filament, respectively.](http://aem.asm.org/)
site-specifically replaced by ΔfliD::D3 for expression of D3/ΔFliD from the chromosome in strain MKSD3. The correct genotype of MKSD3 was confirmed by Southern hybridization using a PCR fragment encoding D1, D2, and D3 as a probe. When the ΔfliC gene of MKSD3 was complemented with pFliCMG1655, filament formation was restored (Fig. 4) and the chromosomal expression of D3/ΔFliD at the filament tip could be detected by immunoelectron microscopy using fibronectin, antifibronectin, and colloidal gold-conjugated protein A (data not shown).

FIG. 5. Analysis of flagella and FliD expressed by the various E. coli recombinant strains. (A) Immunoelectron microscopy analyses of hybrid flagella. The flagella originated from the recombinant strains MKS1(pD3/FliDΔ14) (panel a), MKS1(pFliD) (panel b), MKS2 (pD1D2D3/FliCΔH7) (panel c), MKS2(pFliCΔ7) (panels d, f, h, j, l, and n), MKS1(pSlpA96-245/FliDΔ14) (panel e), and MKS3d(pSlpA96-245/FliCΔH7) (panels g, i, k, and m). Flagella were incubated with soluble fibronectin, antifibronectin antibodies, and colloidal gold-conjugated protein A (panels a to d and g and h), rabbit anti-SlpA antibodies and gold-conjugated protein A (panels c and f and i and j), and monoclonal anti-YadA and gold-conjugated secondary antibodies (panels k and l). Flagella double stained with monoclonal anti-YadA and gold-conjugated secondary antibodies (10 nm in diameter) as well as rabbit anti-SlpA antibodies and gold-conjugated secondary antibodies with a diameter of 5 nm are shown in panels m and n. Black arrowheads indicate binding of anti-SlpA antibodies at the tip (panel c) or along the flagellum (panel m), white arrowheads indicate binding of anti-YadA antibodies along the filament (panel m), and arrows show flagellar hooks (panels g and k). Note the localization of D3 repeats at the tip as well as YadA and SlpA fragments along the flagellar side in strain MKSD3(pSlpA96-245/FliCΔH7)(pYadA84-385/FliCΔH7). Note also the empty hooks. Fn, plasma fibronectin. Antibodies against plasma fibronectin, SlpA96-245, and YadA are indicated by anti-fn, anti-SlpA, and anti-YadA, respectively. Size bars, 200 nm. (B) Western blot with antibodies against a His-tagged D1-D3 peptide (left panel) and anti-SlpA antibodies (right panel) of hybrid flagella originating from the recombinant strains indicated above the panels. Strain MKS2 is a fliC deletion derivative, MKSD3 is a derivative of MKS2 that expresses D3/FliDΔ from the chromosome, and MKS1 is a fliD deletion derivative. A molecular size marker in kDa is indicated on the left.
Plasmids pSlpA96-245/FliC172A and pYadA84-385/FliC172A encoding the N-terminal fragment of SlpA and a fragment of the YadA adhesin (amino acids 84 to 385) from *Yersinia enterocolitica* (7, 21, 27) in the variable region of FliC172, were transformed into MKS3D. The coexpression of D3/ΔFliD at the filament tip (Fig. 5A, panel g) and the hybrid flagellins SlpA96-245/FliC172A and YadA84-385/FliC172A along the filament was detected by immunoelectron microscopy as shown in Fig. 5A, panels i, k, and m. The flagella of the control strain MKS2(pFliC172) carrying wild-type FliC and FliD did not bind fibronectin at the tip, nor did they react with anti-SlpA and anti-YadA antibodies along the filament (Fig. 5A, panels h, j, l, and n).

The results obtained by immunoelectron microscopy were verified by Western blot analysis (Fig. 5B). Crude extract of strain MKS3D was analyzed with antibodies against a His-tagged D1-D3 peptide (12). The strain does not express flagellin but expresses D3/His-tagged D1-D3 peptide (12). The strain does not express anti-SlpA and anti-YadA antibodies along the filament (Fig. 5A, panels h, j, l, and n).

DISCUSSION

The flagellar capping protein FliD is a versatile protein that promotes flagellin assembly into the filament by a specific rotating mechanism (25, 28). FliD also functions as a cap that prevents FliC subunits from diffusing out of the cell. Additionally, in *Pseudomonas aeruginosa* and *Clostridium difficile*, FliD has been reported to function as a mucin-specific adhesin (18, 24).

We show for the first time that the capping protein FliD of *E. coli* can be used as a carrier of foreign peptides at the tip of the flagellum at a distance from the cell surface. The D repeats were inserted at four different sites of fliD by direct insertion into existing restriction sites or by deletion and subsequent insertion. Complementation of the ΔfliD gene in strain MKS1 with the various fliD fusions showed formation of flagellar filaments with variable efficiency depending on which site in fliD had been used for insertion. The variable region in FliD that corresponds to amino acids 189 to 221 tolerated deletion of 14 amino acids and subsequent insertion of the D3 repeat of 39 amino acids. The D3/ΔFliD hybrid was displayed in a fibronectin-binding form at the tip of the flagellum and the strain expressed flagella well. When the site corresponding to amino acid 326 of FliD was used for insertions, flagella were expressed only at a low level. Deletion and/or insertion at other sites resulted in poor complementation of ΔfliD and reduced filament assembly in *E. coli* MKS1.

It was concluded that the variable region at amino acids 205 to 219 was suitable for FliD display and hence it was used for further experiments. This site in FliD is apparently located in the surface-exposed plate structure of the cap (Fig. 1). The regions in FliD that did not efficiently function as deletion and/or insertion sites may be buried within the cap structure. Alternatively, mutations in these sites may disturb FliD secretion and FliC assembly and thereby impair the formation of flagella (14, 25, 28).

FliD also allows display of other peptides longer than the D3 repeats, as expression of FliD fused to the N-terminal 150 amino acids of the *L. brevis* SlpA protein led to the synthesis of typical long flagellar filaments. Finally, we showed that trihybrid flagella can be constructed by coexpression of peptides simultaneously along the flagellar filament and at the filament tip. The *E. coli* strain MKS3D(pSlpA96-245/FliC172A)(pYadA84-385/FliC172A) expressed hybrid D3/ΔFliD from the chromosome at the tip and plasmid-encoded hybrid flagellins carrying the N-terminal SlpA fragment (150 amino acids) as well as a fragment (302 amino acids) of the YadA adhesin of *Y. enterocolitica* along the filament.

The results presented here provide information about protein regions in FliD and support the model of the FliD cap obtained by high-resolution electron microscopy (14, 28). We characterized regions of FliD that allow deletions and insertions and thus can be used in surface display. We also recognized regions that may be involved in the capping mechanism and polymerization of FliD as these sites did not allow manipulations without effects on the formation of flagella.

The trihybrid flagellum display described here can be used in various ways depending on the application. Flagellin fusion proteins efficiently induce strong and specific immune responses against the peptide of interest (4), indicating that trihybrid flagella are potential candidates as antigen carriers in the development of vaccines. FliD display could be used in the construction of oral vaccines to target the desired FliC-displayed antigens to the epithelial cell surface. Our results also indicate that hybrid FliD can be expressed on the cell surface in the absence of FliC apparently directly attached to the hook cap. This may be important in vaccine applications in which FliC-mediated immune response is a disadvantage, e.g., when the displayed antigens are weak immunogens. As an additional application, peptides identified as motifs for entry into eukaryotic cells (23) could be fused to FliD for intracellular delivery of several different effector molecules displayed as FliC fusions along the filament.

ACKNOWLEDGMENTS

We thank Raili Lameranta for technical assistance. Electron microscopy was carried out at the Electron Microscopy Unit, Institute of Biotechnology, University of Helsinki.

This work was supported by the National Technology Agency (Tekes, grant number 40312/03), University of Helsinki (grant number 121300), and the Academy of Finland (grant numbers 78141, 105824, 200290, and 211300 and the Microbes and Man program).

REFERENCES


