Chimeric FimH adhesin of type 1 fimbriae: a bacterial surface display system for heterologous sequences

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The FimH adhesin of type 1 fimbriae has been tested as a display system for heterologous protein segments on the surface of *Escherichia coli*. This was carried out by introduction of restriction site handles (*Bgl*II sites) in two different positions in the *fimH* gene, followed by in-frame insertion of heterologous DNA segments encoding two reporter sequences. In the selected positions such insertions did not significantly alter the function of the FimH protein with regard to surface location and adhesive ability. The system seemed to be quite flexible, since chimeric versions of the FimH adhesin containing as many as 56 foreign amino acids were transported to the bacterial surface as components of the fimbrial organelles. Furthermore, the foreign protein segments were recognized by insert-specific antibodies when expressed within chimeric proteins on the surface of the bacteria. The results from this feasibility study point to the possibility of using the FimH adhesin as a general surface display system for sizeable protein segments.

Keywords: authentic surface display, FimH adhesin, type 1 fimbriae

INTRODUCTION

An attractive way of assaying heterologous peptide segments for biological activity is to fuse them into a naturally occurring bacterial surface protein which has the ability to accept grafts of exogenous protein segments. If the peptide segment is displayed in an immunologically active form, this may be assayed directly on the surface of the bacteria. Here we have used a structural element from a bacterial organelle system, fimbriae, for this purpose. Fimbriae, which are bacterial adhesins that enable bacteria to target to and to colonize specific host tissues, are long thread-like surface organelles, found in up to about 500 copies per cell (for reviews see Hultgren *et al.*, 1993; Klemm & Krogfelt, 1994; Krogfelt, 1991).

A large variety of fimbriae are known and several have been extensively characterized, such as type 1 fimbriae. A

single type 1 fimbria is a thin, 7 nm wide and approximately 1 µm long, surface polymer. It consists of about 1000 subunits of a major building element, i.e. the FimA protein, stacked in a helical cylinder (Brinton, 1965). Additionally, a few minor components, viz. the FimF, FimG and FimH proteins, are also present as integral parts of the fimbriae (Abraham *et al.*, 1987; Hanson *et al.*, 1988; Klemm & Christiansen, 1987; Krogfelt & Klemm, 1988). The 30 kDa FimH protein has been shown by direct and indirect tests to be the actual receptor-binding molecule (for reviews covering this aspect see Hasty *et al.*, 1994; Klemm & Krogfelt, 1994). The FimH adhesin binds to D-mannosides, however, highly conserved variants, differing in a few amino acids, have been shown additionally to bind to protein targets (Sokurenko *et al.*, 1992, 1994). The major component is *per se* not necessary for expression of an adhesive phenotype, since recombinant bacteria unable to produce this component are still able to locate the FimH adhesin extracellularly in a biologically active form compatible with receptor recognition (Klemm *et al.*, 1990).

Like the other components of type 1 fimbriae the FimH adhesin is encoded in the *fim* gene cluster. This encompasses nine genes contained within a 9.5 kb DNA segment

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Abbreviations: DICM, differential interference contrast microscopy; FITC, fluorescein isothiocyanate.

Fig. 1. Overview of plasmids used in this study; only relevant non-vector sectors are shown. (a) The *fim* gene cluster as present in plasmid pPKL4 is shown. The triangle indicates the position of the translational stop-linker in the *fimH* gene in plasmid pPKL115. (b) Plasmid pLPA22 and derivatives are shown. The positions of heterologous inserts are indicated (black boxes). Small triangles indicate signal-peptide-encoding sectors.

Fig. 2. Biosynthesis of type 1 fimbriae. The major structural component, FimA (shaded), minor components FimF and FimG, and the FimH adhesin, are translocated across the cytoplasmic membrane via the normal export machinery. Fimbrial subunits emerging on the periplasmic side of the membrane are complexed by FimC until they arrive at the assembly platform/usher, FimD. The position of a heterologous epitope is indicated on each FimH component by a black dot.

(Fig. 1). We have studied the *fim* genes responsible for type 1 fimbriation of *Escherichia coli* K12 strain PC31 and the biosynthesis of these adhesive organelles as summarized in Fig. 2. The structural components of type 1 fimbriae are produced as precursors having an N-terminal signal sequence. Thus, the FimH protein is produced as a

precursor of 300 amino acids that is processed into a mature form of 279 amino acids (Hanson *et al.*, 1988; Klemm & Christiansen, 1987). All evidence suggests that this translocation is dependent on the normal *E. coli* export system, viz. SecA, SecB, etc. (Dodd & Eisenstein, 1984; Hultgren *et al.*, 1993; Pugsley, 1993). However, further export from the periplasm and across the outer membrane is dependent on a fimbriae-specific export and assembly system constituted by the FimC and FimD proteins (Jones *et al.*, 1993; Klemm, 1992; Klemm & Christiansen, 1990).

The biogenesis machinery of type 1 fimbriae has been shown to be quite tolerant in integrating similar but heterologous structural components into fimbrial organelles. Thus, minor components from F1C fimbriae exhibiting as little as 34 % identity with their equivalents in type 1 fimbriae are nevertheless readily integrated into type 1 fimbriae, resulting in hybrid organelles (Klemm *et al.*, 1994). Also, like the adhesin of P-fimbriae, PapG (Haslam *et al.*, 1994), the FimH protein seems to consist of two major domains, each constituting roughly one half of the molecule; the N-terminal domain seems to contain the receptor-binding site whereas the C-terminal domain seems to contain recognition sequences for the transport machinery (P. Klemm and others, unpublished data). This information suggested that engineering of the FimH adhesin to contain heterologous sequences representing foreign epitopes would be possible without affecting compatibility with the transport system or adhesive ability.

In this study we have used two different positions in the FimH adhesin for insertion and display of heterologous sequences representing the preS2 sector of the hepatitis B surface antigen and an epitope from cholera toxin. We also show them to be exposed in immunologically active forms on the surface of the chimeric FimH adhesins, which in turn are present on the surface of bacterial hosts.

METHODS

Bacterial strain and growth conditions. A variant of the *E. coli* K12 strain HB101 (F' *lacI* Tn5) (Boyer & Roulland-Dussoix, 1969) was used in this study as a host for expression of chimeric fimbriae. This strain is phenotypically Fim⁻ due to a deletion in the *fim* gene cluster (Blomfield *et al.*, 1991). Cells were grown on solid medium or liquid broth supplemented with the appropriate antibiotics. When required gene expression from the *lac* promoter was ensured by addition of the gratuitous inducer IPTG to the growth medium.

Plasmids. Plasmids pPKL4 (all *fim* genes) and pPKL114 (*fimH*) have been described previously (Klemm *et al.*, 1994, 1985). pPKL115 was made by substituting the pBR322 vector part of plasmid pPKL114 with pACYC184 (Chang & Cohen, 1978) by *HindIII*/*EagI* digestion and religation. Plasmid pSM782 (generously provided by S. Molin, Department of Microbiology, Technical University of Denmark), containing the preS2- and S-encoding regions of the hepatitis B viral genome, was made from plasmid λ -HBV1 (Charnay *et al.*, 1979) by subcloning an *EcoRI*-*DraI* fragment into pBR322. Plasmid pLPA22 was made by inserting a 1018 bp *PvuII*-*MluI* fragment containing the *fimH* gene from pPKL4 into plasmid pUC18. The insert was positioned downstream of the *lac* promoter residing in the

Table 1. Summary of plasmids (A, B and U indicate pACYC184-, pBR322- and pUC18-based vector) used in this study with respect to genotype and phenotype when present in strain HB101

Plasmid	Relevant genotype	Insert position	Haemagglutination*
pPKL4 (B)	All <i>fim</i> genes		15
pPKL115 (A)	<i>fimH</i>		> 600
pLPA22 (U)	<i>fimH</i> ⁺		> 600
pLPA29 (U)	<i>fimH</i> -Bg/II	258	> 600
pLPA30 (U)	<i>fimH</i> -Bg/II	225	> 600
pLPA37 (U)	<i>fimH</i> -preS2	258	> 600
pLPA38 (U)	<i>fimH</i> -preS2	225	> 600
pLPA93 (U)	<i>fimH</i> -cholera	225	> 600
pLPA95 (U)	<i>fimH</i> -cholera	258	> 600
pLPA22 (U)+pPKL115 (A)	<i>fimH</i> ⁺ <i>fimH</i>		10
pLPA29+pPKL115	<i>fimH</i> -Bg/II <i>fimH</i>		7
pLPA30+pPKL115	<i>fimH</i> -Bg/II <i>fimH</i>		8
pLPA37+pPKL115	<i>fimH</i> -preS2 <i>fimH</i>		200
pLPA38+pPKL115	<i>fimH</i> -preS2 <i>fimH</i>		90
pLPA93+pPKL115	<i>fimH</i> -cholera <i>fimH</i>		11
pLPA95+pPKL115	<i>fimH</i> -cholera <i>fimH</i>		16

* Haemagglutination of guinea-pig erythrocytes indicated in seconds before reaction occurred. The mean values of four measurements are given.

vector part of the plasmid. Plasmids pLPA29 and pLPA30 were made by inserting 9-mer asymmetric *Bgl*II linkers into the *Bsa*AI and *Hind*III sites, respectively, in the *fimH* gene of plasmid pLPA22. Plasmids pLPA37 and pLPA38 resulted from insertion of a 162 bp PCR fragment (see later), encoding the preS2 region of the hepatitis B virus surface antigen, into the *Bgl*II sites of plasmids pLPA29 and pLPA30, respectively. Plasmids pLPA93 and pLPA95 were made by inserting a 51 bp synthetic double-stranded DNA segment, encoding amino acids 50–64 of the B subunit of cholera toxin and containing a *Bgl*II overhang at one end and a *Bam*HI overhang at the other, into the *Bgl*II sites of plasmids pLPA30 and pLPA29, respectively (see Figs 1 and 3, Table 1).

DNA techniques. Isolation of plasmid DNA was carried out by alkaline extraction (Birnboim & Doly, 1979). Restriction endonucleases were used according to the manufacturer's specifications (Biolabs). DNA sequencing was carried out by the dideoxy chain-termination technique (Sanger *et al.*, 1977) using a Sequenase version 2.0 kit from USB. Oligonucleotides were made at the department's core facilities.

PCR methodology. PCR was performed on a Perkin Elmer Cetus DNA Thermal Cycler 480. Reactions were set up as 100 µl volumes containing 200 µM each of dATP, dCTP, dGTP and dTTP, 0.2–1.0 µM of each of the two primers, 2 mM MgCl₂, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2.5 units AmpliTaq DNA polymerase and 0.1–0.2 µg plasmid template. The reactions were run for 25–30 cycles each consisting of 1 min at 94 °C, 1 min at 40 °C, and 1 min at 72 °C. For amplification of the preS2 fragment the following primers were used: 5'GGAGATCTAATTCCACAACCTT3'; 5'GGAGATCTGTTTCAGCGCAGGGT3'.

Haemagglutination. The capacity of bacteria to express a D-mannose-binding phenotype was assayed by their ability to agglutinate guinea-pig erythrocytes on glass slides. Aliquots of liquid cultures grown to an optical density of 3.0 and 5 % (v/v)

suspensions of erythrocytes were mixed, and the time until agglutination occurred was measured.

Antisera. Rabbit anti-type-1-fimbrial serum raised against purified type 1 fimbriae has previously been described by Krogfelt & Klemm (1988). A monoclonal antibody directed against FimH (Chanteloup *et al.*, 1991) was kindly provided by Dr Maryvonne Dho-Moulin, Institut National de la Recherche Agronomique, France. Goat serum raised against the cholera toxin B subunit (international standard for WHO no. 12–246) was produced at and kindly provided by the State Serum Institute, Copenhagen, Denmark. A monoclonal antibody directed against the preS2 domain of hepatitis B surface antigen (Itoh *et al.*, 1986) was kindly provided by Dr Makoto Mayumi, Jichi Medical School, Japan. Fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit, anti-mouse or anti-goat sera were provided by Dakopatts.

Fixation of bacterial cells and fluorescence labelling. Cells from overnight cultures (induced if required) were harvested and washed in PBS (0.145 M NaCl; 0.15 M sodium phosphate). Cells were fixed by mixing 250 µl cells with a 750 µl 4 % (w/v) solution of paraformaldehyde in PBS. This mixture was incubated on ice for 20 min. To remove the fixative, cells were washed twice in PBS. Samples of 20 µl were placed on a poly-L-lysine-coated slide and air-dried. After washing in PBS, 16 µl of a 1:5 (monoclonal) or 1:25 (polyclonal) dilution of the primary antiserum was placed on top of each sample and left in a moist incubation chamber for 1 h. The slides were washed three times in PBS and 16 µl FITC-conjugated antiserum was added. After 2 h in the dark, the slides were washed three times in PBS, and a drop of Citifluor was placed on top of each sample before microscopy.

Microscopy and image analysis. An Axioplan epifluorescence microscope (Carl Zeiss) equipped with a 100 W mercury burner and filter set 10 (Carl Zeiss) was used to visualize FITC-tagged antibodies. A 63 × 1.25 Plan Neofluar (Carl Zeiss) oil objective

for differential interference contrast microscopy (DICM) was employed. The microscope was equipped with a slow-scan charge coupled device (CCD) camera for capturing digitized images. The CCD camera was a CH250 camera (Photometrics) with a KAF 1400 chip (pixel size 6.8 by 6.8 µm) operated at -40 °C which was read out in 12 bits (4096 intensity levels) at a rate of 200 kHz. The integration time for the CCD camera was 2 s. The bit range of interest, as far as possible the same rate for all images, was linearly scaled to 8-bit files in the PMIS software (version 2.11, Photometrics) and subsequently transferred to Photoshop (Adobe) for final analysis. A DOS-based 486 computer was used as controller for the CCD camera and a Macintosh Quadra 950 was used to run Photoshop.

Electron microscopy. Electron microscopy and immuno-electron microscopy were carried out essentially as described previously by Klemm *et al.* (1994). In short a 25 µl aliquot of bacterial suspension was placed on a carbon-coated, glow discharged grid for 30 s. Grids were washed in 2 drops PBS, dehydrated for 5 min in each of the following concentrations of ethanol, 25%, 50%, 75% and 96% (v/v), blotted dry, and shadowed with tungsten wire at an angle of 30°. For immunoelectron microscopy, a monoclonal antibody directed against the preS2 region was used diluted 1:5 as the primary antibody and rabbit anti-mouse serum conjugated with 10 nm gold particles (Dako) was diluted 1:20 and used as the secondary antibody.

RESULTS

Engineering new restriction sites into *fimH*

In the present study, double plasmid systems have been used. In each pair, one plasmid encoded the *fimH* gene (either a wild-type or an engineered version), whereas the second plasmid encoded the total *fim* gene cluster in which a translational stop-linker had been inserted in the *fimH* gene (Fig. 1, Table 1).

Based on algorithms which predict hydrophilicity and secondary structure we selected two positions in the C-terminal domain of the FimH protein for insertion of heterologous sequences. These correspond to positions 225 and 258 in the mature protein, sites predicted to be situated in a surface-exposed part of the FimH protein. In order to facilitate later manipulations the *fimH* gene was subcloned into the pUC18 vector resulting in plasmid pLPA22. Subsequently, a *Bgl*II site was introduced in-frame into positions 225 and 258, respectively. This was carried out by site-directed mutagenesis employing synthetic oligomers resulting in plasmids pLPA30 and pLPA29, respectively (Fig. 1). The introduced *Bgl*II sites resulted in a codon change from a Leu to a Phe codon in position 225 and either the addition of codons for the sequence Arg-Ser-Ser, in the case of plasmid pLPA29, or addition of codons for the sequence Arg-Ser-Gly in position 258, in the case of plasmid pLPA30. Sequence analysis of the entire modified *fimH* genes in plasmids pLPA29 and pLPA30 confirmed that no other changes had occurred. Host cells which, in addition to plasmid pLPA29 or pLPA30, also contained plasmid pPKL115 (*fimH*), showed wild-type phenotypic characteristics with regard to adhesion and fimbriation as judged by haemagglutination and immunofluorescence microscopy (Table 1).

(a)

***** *Bgl*II

CAG TTC AGA TCT AAT TCC ACA ACC TTC CAC CAA ACT CTG CAA GAT
Gln Phe Arg Ser Asn Ser Thr Thr Phe His Gln Thr Leu Gln Asp
224

CCC AGA GTG AGA GGC CTG TAT TTC CCT GCT GGT GGC TCC AGT TCA
Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala Gly Gly Ser Ser Ser

GGA ACA GTA AAC CCT GTT CTG ACT ACT GCC TCT CCC TTA TCG TCA
Gly Thr Val Asn Pro Val Leu Thr Thr Ala Ser Pro Leu Ser Ser

*Bgl*II

ATC TTC TCG AGG ATT GGG GAC CCT GCG CTG AAC AGA TCT TCG ACG
Ile Phe Ser Arg Ile Gly Asp Pro Ala Leu Asn Arg Ser Ser Thr
226

(b)

***** *Bgl*II

CAG TTC AGA TCT GTT GAA GTT CCG GGT AGT CAG CAT ATC GAT AGT
Gln Phe Arg Ser Val Glu Val Pro Gly Ser Gln His Ile Asp Ser
224

*Bam*HI/*Bgl*II

CAG AAA AAA GCT GGA TCT TCG ACG
Gln Lys Lys Ala Gly Ser Ser Thr
226

Fig. 3. Sectors of chimeric *fimH* genes containing heterologous sequences encoding (a) the preS2 region of the hepatitis B surface antigen (plasmid pLPA38) or (b) a segment of the cholera toxin B chain (plasmid pLPA93). Non-heterologous sequences are indicated by asterisks; sectors mimicking hepatitis B or cholera toxin sequences are underlined. Numbers correspond to the positions of the amino acid residues in the mature FimH protein.

Engineering heterologous DNA sequences encoding reporter peptides into *fimH*

As heterologous reporter peptides we have selected the preS2 region of the hepatitis B surface antigen and a well-characterized region of the B subunit of cholera toxin. The preS2 region is known to contain protective antigenic determinants (Itoh *et al.*, 1986; Pfaff *et al.*, 1986). In addition, this region is disulfide-bond-independent and apparently more immunogenic than the major S protein (Tiollais *et al.*, 1985). The cholera toxin segment consists of residues 50–64 of the B subunit and has previously been shown to elicit antibodies that bind to and neutralize cholera toxin (Jacob *et al.*, 1983).

A DNA segment of 162 nucleotides encoding 52 of the 55 amino acids of the preS2 region was amplified by PCR technology using plasmid pSM782 as template and primers that provided the amplified sequence with flanking *Bgl*II sites. Following restriction with *Bgl*II and purification the amplified fragment was inserted into the *Bgl*II sites of plasmids pLPA29 and pLPA30 resulting in plasmids pLPA37 and pLPA38, respectively (Figs 1 and 3). Subsequent sequence analysis confirmed that the inserts were correctly oriented and that the reading frame of the chimeric *fimH*-preS2 genes was correct (see Fig. 3).

A synthetic DNA segment encoding the cholera epitope was made by annealing two complementary 51 bp oligo-

Fig. 4. Electron micrographs showing levels of fimbriation of *E. coli* HB101 hosts containing the following plasmids: pPKL115 (a), pLPA22 plus pPKL115 (b), pLPA37 plus pPKL115 (c), pLPA38 plus pPKL115 (d). Bar, 0.5 μ m.

nucleotides which were designed to result in a final double-stranded DNA segment with a *Bgl*II overhang at one end, a *Bam*HI overhang at the other, and an internal *Cla*I site. The epitope-encoding segment was inserted into the *Bgl*II site in the *fimH* gene in plasmids pLPA29 and pLPA30, creating plasmids pLPA95 and pLPA93, respectively, resulting in regeneration of a *Bgl*II site at only one end of the insert. This feature was used to identify plasmids with correct orientation of the insert. The presence of the *Cla*I site was used for initial screening for clones containing the insert. Sequence analysis of plasmids pLPA93 and pLPA95 confirmed the orientation and conservation of the reading frame in the chimeric *fimH*-cholera genes (Figs 1 and 3).

Expression of chimeric FimH containing heterologous sequences mimicking the preS2 domain of hepatitis B surface antigen and a cholera toxin epitope

To evaluate whether the heterologous inserts in *fimH* resulted in protein expression we used the T7 polymerase/promoter system (Tabor & Richardson, 1985). Subcloning into the pGEM3 vector system and subsequent assaying revealed that proteins with the expected sizes were produced from the chimeric *fimH* genes in all cases (data not shown). To assess whether the FimH proteins harbouring foreign inserts were accepted by the type 1 fimbrial transport system and, additionally, were present on the bacterial surface in a biologically functional form, we looked at the adhesive phenotypes of recombinant strains expressing the chimeric FimH proteins. Bacterial hosts which, in addition to plasmid pLPA38 (preS2 insert in position 225 in FimH), also

Fig. 5. Assessment of bacterial surface location of FimH variants by DICM and fluorescence microscopy (FM). In all cases a monoclonal antiserum directed against FimH was used as the primary serum and a fluorescence-labelled anti-mouse serum was used as the secondary antiserum (see Methods for further details). (a) DICM and (b) FM of host cells expressing type 1 fimbriae lacking the FimH protein (plasmid pPKL115); (c) DICM and (d) FM of cells expressing wild-type type 1 fimbriae (plasmids pPKL115 plus pLPA22); (e) DICM and (f) FM of cells expressing chimeric type 1 fimbriae where the FimH protein contains the preS2 segment of the hepatitis B surface antigen in position 258 (plasmids pPKL115 plus pLPA37); (g) DICM and (h) FM of cells expressing chimeric type 1 fimbriae where the FimH protein contains the preS2 segment of the hepatitis B surface antigen in position 225 (plasmids pPKL115 plus pLPA38); (i) DICM and (j) FM of cells expressing type 1 fimbriae where the FimH protein contains a cholera toxin epitope in position 225 (plasmids pPKL115 plus pLPA93); (k) DICM and (l) FM of cells expressing type 1 fimbriae where the FimH protein contains a cholera toxin epitope in position 258 (plasmids pPKL115 plus pLPA95). Bar, 5 μ m.

contained plasmid pPKL115 (*fimH*) caused, when induced by IPTG, strong agglutination of guinea-pig erythrocytes, indicative of the presence of a biologically active form of the FimH adhesin on the cells (Table 1). The combination of plasmids pLPA37 (preS2 in position 258 in FimH) and pPKL115 resulted in weaker, but detectable, haemagglutination (Table 1). Furthermore, such cells were fimbriated, albeit less than the wild-type control (Fig. 4).

In the cases where a sequence mimicking a cholera epitope had been inserted into FimH, viz. pLPA93 (insert in

position 225) and pLPA95 (insert in position 258), an agglutination phenotype also resulted when either of these plasmids were complemented by plasmid pPKL115 (*fimH*) (Table 1). In addition to the adherence phenotypes of the various clones, we monitored the presence of engineered FimH adhesins on the surface of the cells by CCD microscopy in connection with fluorescent antibody methodology employing a FimH-specific monoclonal serum (Fig. 5). In all cases, significant signals, albeit of varying intensity, were detected when compared to a negative control strain that harboured the auxiliary plasmid pPKL115 alone.

Fig. 6. Surface display of foreign peptides by FimH assessed by DICM and fluorescence microscopy (FM) of *E. coli* cells. In all cases fluorescence-labelled secondary antisera were used; the type of primary serum employed is indicated (see Methods for further details). (a) DICM and (b) FM of cells expressing wild-type type 1 fimbriae (plasmids pPKL115 plus pLPA22) reacted with anti-preS2 monoclonal antiserum (primary serum); (c) DICM and (d) FM of cells expressing chimeric type 1 fimbriae where the FimH protein contains the preS2 segment of the hepatitis B surface antigen in position 258 (plasmids pPKL115 plus pLPA37) reacted with anti-preS2 monoclonal antiserum (primary serum); (e) DICM and (f) FM of cells expressing chimeric type 1 fimbriae where the FimH protein contains the preS2 segment of the hepatitis B surface antigen in position 225 (plasmids pPKL115 plus pLPA38) reacted with anti-preS2 monoclonal antiserum (primary serum); (g) DICM and (h) FM of cells expressing wild-type type 1 fimbriae (plasmids pPKL115 plus pLPA22) reacted with anti-cholera-toxin serum (primary serum); (i) DICM and (j) FM of cells expressing type 1 fimbriae where the FimH protein contains a cholera toxin epitope in position 225 (plasmids pPKL115 plus pLPA93) reacted with anti-cholera-toxin serum (primary serum); (k) DICM and (l) FM of cells expressing type 1 fimbriae where the FimH protein contains a cholera toxin epitope in position 258 (plasmids pPKL115 plus pLPA95) reacted with anti-cholera-toxin serum (primary serum). Bar, 5 μ m.

Detection of the preS2 segment of the hepatitis B surface antigen and the cholera toxin epitope displayed on chimeric FimH proteins

Since there was good evidence for the chimeric FimH proteins to be present on the surface of the *E. coli* hosts, we proceeded by testing the ability of specific antisera, raised against the preS2 sector of the hepatitis B surface antigen and the cholera toxin B chain, to respectively recognize the chimeric FimH-preS2 and FimH-cholera proteins directly on the surface of the recombinant bacteria. By immunofluorescence microscopy *E. coli* hosts

harbouring either plasmid pLPA37 (preS2 sequence in position 258) or pLPA38 (preS2 sequence in position 225) in addition to plasmid pPKL115 (*fimH*) were shown to react specifically with antisera directed against the inserted heterologous sequence (Fig. 6c–f), whereas hosts expressing wild-type FimH did not (Fig. 6a, b). Similar results were obtained (Fig. 6g–l) with the cholera toxin insert in the same positions (plasmids pLPA93/pPKL115 and pLPA95/pPKL115).

The results obtained by immunofluorescence microscopy were corroborated by immunoelectron microscopy,

Fig. 7. Immunoelectron microscopy with colloidal-gold labelling of *E. coli* HB101 cells containing plasmids pLPA22 plus pPKL115 (a), pLPA37 plus pPKL115 (b) or pLPA38 plus pPKL115 (c), using anti-preS2 monoclonal antiserum. Bar, 0.1 μ m.

employing the preS2-specific monoclonal antibody as primary serum and a colloidal-gold-labelled secondary antiserum. A significant number of gold particles were seen, mostly in connection with the fimbrial organelles, on bacterial hosts harbouring chimeric *fimH*-preS2 genes (Fig. 7b, c), whereas only a few gold particles were present on the control strain expressing wild-type fimbriae (Fig. 7a). Furthermore, in the control, gold particles were not seen to be connected with the fimbriae.

DISCUSSION

The present results illustrate the feasibility of engineering the FimH adhesin of type 1 fimbriae for presentation of heterologous protein segments. In this prototype study we have expressed segments of two important, immunologically relevant proteins within chimeric FimH proteins. Two sites, corresponding to positions 225 and 258 in the mature FimH protein, were used for insertion of foreign reporter sequences. Both positions proved to be compatible with integration of such sequences with regard to production, cell-surface exposure and at least partial conservation of adhesive function of the chimeric FimH proteins. Furthermore, and most remarkably, the inserted reporter sequences, i.e. the preS2 hepatitis segment and the cholera toxin epitope, were displayed on the surface of the FimH proteins in conformations which immunologically mimicked the conformations of the domains in the parental proteins viz. the hepatitis B surface protein and cholera toxin, respectively (Figs 6 and 7).

Bacterial hosts that expressed chimeric FimH proteins exhibited diminished haemagglutination titres compared to a control strain expressing wild-type FimH protein (Table 1). The reduced adhesiveness could be due to two factors; firstly, the foreign inserts might result in reduced amounts of the adhesins reaching the cell surface, perhaps due to unfavourable interference of the heterologous sequences with the fimbrial transport and bioassembly machinery. Alternatively, the chimeric FimH proteins may fold slightly differently than the wild-type version causing changes in the receptor-site domain of the molecule, which could result in lowered binding affinities. Judging from the reduced reaction of the strains expressing chimeric FimH adhesins with a FimH specific monoclonal antibody as compared to a FimH wild-type control (Fig. 5), the first proposed scenario seems most plausible. However, we cannot rule out a concerted effect of both the proposed possibilities. Nevertheless, the data indicate that significant amounts of the chimeric FimH proteins are in fact present on the surface of the recombinant strains. The C-terminal domain of FimH therefore seems to be amenable for integration of heterologous inserts of substantial size. For example introduction of a *Bg*/II linker and a preS2-encoding segment in position 225 in *fimH* (pLPA38) results in addition of 56 foreign amino acids to the FimH protein, of which 52 encode the preS2 segment. This corresponds to a 20% increase in the size of the sequence of the mature FimH adhesin, and was achieved without a dramatic reduction of the normal function of the adhesin.

We have also studied the possibility of using the major structural protein of type 1 fimbriae, FimA, for display of heterologous sequences (Hedegaard & Klemm, 1989; L. Pallesen and others, unpublished). Similar studies have been carried out employing major structural proteins of other fimbrial species for this purpose. However, one of the conclusions from these investigations (as reviewed by Pallesen & Klemm, 1994) is that none of these systems seem to be able to accept foreign inserts larger than about 25 amino acids without severely affecting the function of the proteins. The FimH protein seems to be more flexible in this respect, and with its ability to display heterologous sequences of more than 50 amino acids it could constitute a valuable additional system.

The FimH protein only constitutes a small percentage of the total fimbrial protein. However, over-production of the FimH protein has been achieved (Abraham *et al.*, 1988; H. Hasman & P. Klemm, unpublished results) and this can probably also be realized for chimeric versions of the adhesin.

The ability of the FimH protein to display two completely different heterologous protein segments such as the preS2 domain of the hepatitis B viral surface antigen and a segment from the cholera toxin B chain on the surface of *E. coli* hosts is indicative of the tolerance of the system and points to several future applications. One obvious possibility is to use chimeric FimH proteins for presentation of immunologically relevant epitopes for vaccine purposes. The data presented on the ability of the system to display large foreign segments in immunologically authentic forms seems promising for such an approach. Furthermore, although the reporter sequences we have used in the present study represent immunologically relevant protein domains, there should also be several other potential applications. A possibility is to use FimH to display segments that represent the receptor-binding domains of naturally occurring adhesins, enzymes, or other target-recognition proteins. This could form the basis for an efficient and rational way of studying protein-protein interaction and receptor recognition in general. Also, one could perhaps envisage the system to be used for the display of randomly created peptide libraries. In connection with modern panning techniques, such a strategy would allow for the selection of interesting novel target-recognition chimeras.

In conclusion, we believe that the FimH presentation system has a large intrinsic potential: firstly, because it can confer surface display of immunologically active heterologous protein segments of substantial size; secondly, since fimbriae, and thereby FimH, are easy to purify it should be possible to study chimeric versions not only *in situ* in bacteria but also as isolated proteins.

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